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수의학박사학위논문

**Oxygen tension and Antioxidants  
on development of porcine *in vitro*  
produced embryos**

산소분압 및 항산화제에 의한  
돼지 체외배아 발육 향상에 관한 연구

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수의학과 수의산과·생물공학 전공

강 정택

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# **Oxygen tension and Antioxidants on development of porcine *in vitro* produced embryos**

**By JungTaek Kang**

**A THESIS SUBMITTED IN PARTIAL  
FULFILLMENT OF THE REQUIREMENT FOR  
THE DEGREE OF DOCTOR OF PHILOSOPHY**

**in**

**Theriogenology and Biotechnology  
Department of Veterinary Medicine, Graduate School  
Seoul National University**

**We accept this thesis as confirming to the required standard**

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**Seoul National University**

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## *Declaration*

*This thesis is submitted by the undersigned for the examination for the degree of Doctor of Philosophy to Seoul National University. This thesis has not been submitted for the purpose of obtaining any other degree or qualification from any other academic institution.*

*I hereby declare that the composition, work and experiments of this thesis are entirely my own.*

*JungTaekKang*

## **ABSTRACT**

# **Oxygen tension and Antioxidants on development of porcine *in vitro* produced embryos**

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Because of availability of pigs in biomedical research, *in vitro* production of porcine embryos is very crucial step. The oxygen toxicity resulted from ROS on *in vitro* environment could be one among numerous causes of low *in vitro* productivity. The purpose of this study is to investigate effects on embryo culture as change of culture condition resulted from different oxygen tension and to increase *in vitro* production rates of porcine embryos by supplementing some antioxidants to the media for defending of oxygen toxicity. So this study was conducted to investigate the effect of different oxygen concentration (5 and 20%) during *in vitro* maturation (IVM) and *in vitro* culture (IVC) and gene expression pattern, and to investigate effects of some antioxidants such as melatonin,

flavonoids (quercetin and taxifolin) on porcine oocyte maturation and embryo further development.

First, as a result of the effect of two oxygen concentrations during IVM and IVC on porcine embryo development, there were no significant differences in oocytes nuclear maturation rate. However, on further culture in 20% IVM, the 5% IVC group showed significantly increased blastocyst formation rate compared to the 20% IVC group. According to mRNA abundance data of multiple genes, each treatment altered the expression of genes in different patterns. As a result, in low oxygen, it occurred with a higher glucose uptake and an increase in anaerobic glycolysis in the cumulus cells, whereas in high oxygen, it happened to a higher activity of mitosis-promoting factor and antioxidant response in cumulus cells. Therefore, it can be concluded that high oxygen concentration during IVM and low oxygen during IVC may alter the expression of multiple genes related to oocyte competence and significantly improves embryo development.

Second, as one of means to supplement of antioxidants, melatonin was added to maturation media. Melatonin at the 10 ng/ml concentration during maturation showed the beneficial effect on the maturation rate and further developmental competence and lower levels of ROS. Also, as results of the local expression of the endogenous melatonin, melatonin receptor-1 gene expressed in cumulus and granulosa cells surrounding on oocytes. I concluded the exogenous melatonin has beneficial effects on nuclear and cytoplasmic maturation during porcine IVM. But

it is not clear whether the observed effects may be mediated by receptor binding or receptor independent, as a direct free radical scavenging.

The result of some flavonoids (quercetin and taxifolin) treatment as other antioxidants showed all quercetin and taxifolin treatment did not improve nuclear maturation of oocytes, but a significantly greater proportion of parthenogenetically activated oocytes developed into blastocysts when the IVM medium was supplemented with adequate quercetin (1 $\mu$ g/ml). As measurement result of levels of ROS and GSH in oocytes and embryos produced in maturation medium supplemented with quercetin or taxifolin, both treatment groups had significantly lower levels of ROS than controls, however GSH levels were different only in quercetin treated oocytes. I concluded that exogenous flavonoids such as quercetin reduce ROS levels in oocytes and may work effectively on embryonic development.

In conclusion, I suggested that low oxygen tension during culture effectively contribute to *in vitro* embryo development of porcine oocytes and that media supplemented with exogenous antioxidant such as melatonin and quercetin on the maturation process of porcine oocyte could promote maturation rate *in vitro* by reducing ROS level arisen during *in vitro* culture.

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Keywords : porcine oocyte, *in vitro* maturation, *in vitro* culture, antioxidant, oxygen tension, ROS

**Student number : 2005-23754**



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## LIST OF ABBREVIATIONS

<b>6-DMAP</b>	6-dimethylaminopurine
<b>BSA</b>	Bovine Serum Albumin
<b>COC</b>	Cumulus-oocyte complex
<b>DPBS</b>	Dulbecco's Phosphate Buffered Saline
<b>EGF</b>	Epidermal growth factor
<b>FBS</b>	Fetal bovine serum
<b>FSH</b>	Follicle stimulation hormone
<b>HEPES</b>	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
<b>ICSI</b>	IntraCytoplasmic Sperm Injection
<b>IVC</b>	<i>In vitro</i> culture
<b>IVM</b>	<i>In vitro</i> maturation
<b>LH</b>	Luteinizing Hormone
<b>PBS</b>	Phosphate Buffered Saline
<b>PCR</b>	Polymerase Chain Reaction
<b>PFF</b>	Porcine Follicular Fluid
<b>PVA</b>	Polyvinyl Alcohol
<b>PZM-3</b>	Porcine Zygote Medium-3
<b>ROS</b>	Reactive Oxygen Species
<b>RT</b>	Reverse Transcript
<b>SCNT</b>	Somatic Cell Nuclear Transfer
<b>TALP</b>	Tyrode's albumin lactate pyruvate
<b>TCM</b>	Tissue Culture Medium

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# **PART I**

## **GENERAL INTRODUCTION**

# **1. LITERATURE REVIEW**

## **1.1 Pig as a scientific resource animal**

Recently, pigs have increasingly become utilized as biomedical research models. This increased use as an animal model is not only a result of regulatory pressure on other animal species such as rodents and primates, but also because pigs are recognized as a suitable animal model for human disease based upon their similarity in aspect of anatomy and physiology.

As advantages of pigs compared to other animal models, pigs are highly reproductive displaying early sexual maturity with 5~8 months, a short generation interval of 12 months, parturition of multiple offspring, an average of over 10 piglets per litter, and all season breeding [1]. Nowadays, standardization of the environment such as pig housing, feeding, and hygiene management, is well developed [2]. Also, reproductive technology and techniques of genetic modification have considerably advanced recently. Therefore, pigs are considered as pretty useful laboratory animal.

The human and pig have a large number of similarities in anatomy, physiology, metabolism, and pathology. Over the last a few decades, pigs have replaced other animal models as the general surgical model in the area for both training and research. Specially due to both anatomical and physiological similarity, the pig has been used successfully in studies of the cardiovascular system [3]. Pigs are used as

general cardiovascular surgical models including atherosclerosis. Also, because the physiology of digestion in omnivorous swine is remarkably like that in humans, pigs have become an increasingly important animal model for pharmaceutical applications for gastrointestinal system [4]. Furthermore, although porcine insulin differs from human insulin by only one amino acid, pig islet cells are functionally similar to humans, so pig islet may transplant to diabetes-patients [5]. Alike a rat, mouse, and rabbit, pig skin has been shown to be the most similar to human skin [6]. Pig skin is structurally similar to human epidermal thickness and dermal-epidermal thickness ratios, so they are especially useful in studying wound healing and burn lesions [7]. Like this in many human disease studies, pigs have been used for a long time.

Mainly, livestock pig breeds and miniature pigs are relevant models in many fields of medical research [8]. But there exist some differences between domestic farm breeds and miniature breeds. The differences are related to their growth rate and size at sexual maturity rather than actual anatomic differences in organs and structures. Intense breeding efforts have provided pig breeds differing substantially in important traits such as size, metabolic characteristics, and behavior. Standard farm pigs could be used in biomedical research protocols, but their use is typically limited to short-term studies because of their rapid growth rate, high feed intake, and large adult weight (280~450 kg). Specially bred-for-research minipigs are available from several sources and have proven to be valuable research animals due to the relatively slow growth rate and decreased adult weight (70 to 120 kg). Some minipig breeds that are currently available include the Yucatan, Hanford, Sinclair,



and Pitman-Moore. Taken together, it makes minipigs easier to use in studies more than farm pigs.

Moreover, if livestock pig breeds are employed for experimentation, the genetic background is mostly not defined. In contrast, minipig outbred stocks with full pedigree are delivered from commercial suppliers. In addition, inbred minipigs are available [9, 10]. Some pig breeds are used as non-rodent models for pharmacological and toxicological studies and are fully accepted by regulatory authorities worldwide.

The evolutionary gap between human and animal models such as rodents has always interfered with an applicability of information gained from many animal experiments to the human therapy. In this regard, the pig model offers many advantages [11]. In other words, the pig is a member of the artiodactyls (cloven-hoofed mammal) which are evolutionarily distinct from the primates and rodents [12], but there is extensive conserved homology between the pig genome and the human genome. The first draft of the porcine genome was generated by the “Sino-Danish Pig Genome Project” in 2005 using shotgun sequencing [13]. This initial evolutionary analysis based on 3.84 million shotgun sequences (0.66X coverage) from the pig genome and the available human and mouse genome data revealed that for each of the types of orthologous sequences investigated (e.g., exonic, intronic, intergenic, 5' UTR, 3' UTR, and miRNA), the pig is much closer to human than mouse [13]. This was confirmed by the comparative analysis of protein coding sequences using full-length cDNA alignments comprising more than 700 kb

from human, mouse, and pig where most gene trees favored a topology with rodents as outgroup to primates and artiodactyls [14]. To date, the first high coverage of the pig from a Duroc sow genome (*sus scrofa*) has been released through Ensembl ([http://www.ensembl.org/Sus\\_scrofa/Info/Index](http://www.ensembl.org/Sus_scrofa/Info/Index)) [15]. According to this Ensembl information, the genome of the pig comprises 18 autosomes, with X and Y sex chromosomes. The genome size is similar to that of human at around 2.7 Gb.

Finally, pigs are increasingly used as animal models for biomedical research due to the medical needs. The severe shortage of available donor organs (e.g., heart, kidney or liver) for organ transplantation has become a life threatening problem for patients with organ failure or organ damage, so making pig-to-human transplantation have come to the fore as an obvious solution for this problem [16]. But there are several obstacles to be overcome before this goal can be achieved. Preclinical studies which porcine heart or kidney was transplanted to primates showed that they created broadly complex rejection responses [17]. Also, pigs have emerged as potential sources of islet transplantation for clinical purpose. Wild-type porcine islets transplanted into the portal vein have successfully reversed diabetes in nonhuman primates [18]. But because of incomplete result of porcine islet transplant, there have been studies by using genetic engineered porcine model to overcome a few obstacles [19]. In addition, the pathogenesis, prevention and treatment of many human diseases (e.g., Alzheimer's disease, breast cancer, cardiovascular diseases) are still poorly understood while the incidences of those diseases are increasing yearly. The use of currently available rodent models for

studying human diseases is in some cases limited due to the many obvious differences between rodents and humans. Pigs are thus becoming an alternative animal model for studying human diseases [20].

Taken together, pigs as a scientific research model were developed to provide many investigators, veterinarians, technicians and others with the best possible medical source for biomedical purposes. As this resource expands gradually, the progressive development of human medical care will be approached in the near future.

## **1.2. *In vitro* maturation system of porcine oocytes**

The development of new techniques in pig reproduction such as transgenesis and cloning, creates a large demand for oocytes and embryos. Thus oocyte maturation is a critical component of IVP of embryos. *In vitro* maturation of oocytes is conducted above all things to gain the oocytes available to use other multiple procedures *in vitro* such as IVF, ICSI and SCNT. The goal of IVM is to get the *in vitro* oocytes progressed from the diplotene stage of prophase I (germinal vesicle or GV) to metaphase II (M II), along with cytoplasmic maturation that encompasses a broad set of intracellular events, all of which are essential for the fertilization and early development of the embryo [21]. Oocytes recovered from ovaries collected at the slaughter house were on immature state in both nuclear and cytoplasmic aspects. So it is important for immature oocytes to be matured to conduct further developmental process and usage. Since pig oocytes can be matured and this IVM oocytes can be fertilized *in vivo* [22], piglets were gained successfully from IVM-IVF oocytes [23]. But, although a great deal of progress has been made during last several decades, this current IVM systems still suffer from major several problems. First, it exist a low rate of development of IVM oocytes to the blastocyst stage and their low quality compared with *in vivo* produced embryos. Also in IVF process, a low rate of male pronucleus formation and a high rate of polyspermy in porcine oocytes [24].

It is well-known that the developmental competence of oocytes derived from small follicle (<3mm in diameter) to reach the M II stage after IVM is lower than

that of oocytes from medium follicle (3~6mm) [25]. According to recent research to examine the comparative competence of mature oocytes aspirated from small follicles and medium follicles of slaughterhouse-derived gilt ovaries, oocytes from small follicles are lower on the maturation rate compared to oocytes from medium follicles. But matured oocyte from two follicles had similar fertility ability *in vitro* and relative transcript abundance of concerned genes [26]. So, further studies are needed to determine the exact stage of ovary and the size of follicles on oocyte competence.

In oocyte morphology, pig oocytes form cumulus-oocyte complexes (COCs). Oocytes are selected using a criterion such as their morphology, including the numbers of cumulus cell layers surrounding the oocytes and uniform of the cytoplasm [27]. Normally, COCs with homogeneous ooplasm and a compact cumulus cell mass have been collected among many oocytes recovered from ovaries. COCs selected for collection process sometimes varies in quality and morphology according to subjective investigations. To prevent this asynchronous maturation due to subjective selection, recently the brilliant cresyl blue (BCB) test has been used successfully to select homologous oocytes for IVP [28]. However, according to recent research on chromosomal aberrations in oocytes selected by BCB test, it showed that BCB test rather disturb nuclear maturation of porcine oocytes [29].

Oocytes in culture are affected by specific physical conditions such as osmolarity, ionic composition, temperature, pH, CO<sub>2</sub>, and O<sub>2</sub> tension as well as maturation

media containing diverse supplements. In order to achieve *in vitro* matured porcine oocytes, media supplemented with serum or porcine follicular fluid (PFF), however, they contain many unknown factors and there is often considerable variability among sources or even among batches from the same source. Furthermore, there is a possibility that these fluids may contain hidden viruses [30]. Several reports have demonstrated that it is possible to replace serum or PFF with other defined compounds for maturation medium without reducing the efficiency on IVP embryo development [31, 32]. In the latter study, successful IVP in the defined system using a chemically defined medium was described in pig [33], and successful piglet production has been reported in the defined system [34].

Various basic culture medium types have been used for IVM of pig oocytes including North Carolina State University (NCSU) medium [35], modified tissue culture medium (TCM) 199 and modified Tyrode's medium containing lactate and pyruvate (mTALP) [36]. Many researches have attempted to overcome the low rate of IVM by providing conditions more similar to the *in vivo* maturation. The supplementation of various hormones in IVM medium has been performed and this has shown beneficial effects on oocytes maturation, such as follicle-stimulating hormone (FSH) [37], luteinizing hormone (LH) [38], pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotropin (hCG) [39], estradiol-17 $\beta$  [40], leptin [41] and relaxin [42]. Moreover, increasing studies have investigated the growth factors and many other factors that improve oocyte maturation, such as nerve growth factor (NGF) [43], brain-derived neurotrophic factor (BDNF) [44], growth differentiation factor 9 (GDF-9) [45], TGF- $\alpha$  [46],

epidermal growth factor (EGF) [47], EGF-like peptides [48], complement component 3 (C3) derivatives [49], L-carnitine [50] and sonic hedgehog [51].

Many researches suggest practical ways to solve the problems mentioned above, that is low rate of maturation and quality compared to *in vivo* counterpart.

### 1.3. *in vitro* embryo culture (IVC)

*In vitro* culture of embryos is an important procedure for improving the developmental competence of *in vitro* embryos produced by artificial process such as SCNT, IVF and ICSI. However, compared with *in vivo* production embryo, the overall efficiency of the pig embryo IVP technology is still extremely low and blastocysts obtained after IVF, SCNT and IVC is inferior in cell numbers and their ability to produce alive litters [52]. In addition, IVP embryos are characterized by a higher mean number of nuclei exhibiting DNA fragmentation per blastocyst compared to those produced *in vivo* [53]. Cytogenetic analysis of pig blastocysts produced *in vitro* showed that chromosomal aberrations occurred more higher in these blastocysts compared to embryos obtained *in vivo* [54, 55]. In general, the transfer of mammalian embryos produced *in vitro* results in considerable developmental abnormalities such as increased embryo mortality, extended gestation and considerably greater body weight of progeny [56, 57].

Although there are many causes for the developmental failure of IVP embryos, chromosomal abnormalities appear to be a major cause of this problem. According to study to determine the relationship between embryonic development speed at different stages and incidence of chromosome abnormalities in *in vitro* produced porcine embryo, the chromosomal abnormalities was significantly occurred higher in the blastocyst derived from 2-cell and >8-cell stage embryos than in the blastocyst derived from the other stage embryos. It indicated that selection of the best quality IVP embryos demands evaluation of embryo developmental



competence from early stages to parturition [55].

However, suboptimal culture conditions remain the major factor affecting the yield and quality of embryos obtained *in vitro*. Many culture media have been tested to optimize these conditions [58, 59]. Currently, the most commonly used media have a precise defined chemical composition and, in addition to salts and proteins, they contain energy sources such as glucose, calcium lactate, pyruvate and amino acids. Several media such as NCSU-37 Medium [60], modified synthetic oviduct fluid (mSOF) [61] and porcine zygote medium (PZM) [62] are available for the successful culture of embryos to the blastocyst stage.

Although a defined porcine IVP system has recently been developed by using PVA instead of PFF during IVM [33], but the most common component of culture media is the protein in the form of BSA or FBS. A beneficial effect of serum addition on the development of embryos has been reported in pigs [63]. Porcine embryos cultured with FBS to the blastocyst stage survived cryopreservation better than those that were not ( $P < 0.05$ , 42.9% vs. 28.6% respectively) [64]. And it has reported successful piglet production by IVF of oocytes matured *in vitro* using NCSU-37 supplemented with FBS [65]. Moreover, pig embryos were cultured in NCSU-23 medium supplemented with BSA significantly increased the proportion of morula and blastocysts production and decreased the average number of apoptotic nuclei and DNA fragmented nucleus index of blastocysts as compared to protein-free group (control) [66].

However, the difficulty to standardize culture conditions with the use of serum or serum albumin as well as infection risk have caused a growing interest in the development of medium with a strictly defined chemical composition. The use of these medium might provide more reproducible culture conditions and eliminate the presence of non-specific or pathogenic factors. One study attempted to replace BSA with PVA in adding to PZM media as macromolecular components [62]. In this study, total cell numbers in embryo cultured in PZM with PVA were greater than other media. The results of experiments on *in vitro* development of pig embryos in medium with animal protein substitute are encouraging and prompt further research. Also, addition of amino acids like glutamine and hypotaurine to culture medium promoted the development of embryos in pigs [67, 68].

Some study showed that exogenous supplement into culture condition is available to improve development of *in vitro* cultured embryos and make a higher blastocyst formation rate. For example, vitamin E, the predominant lipid-soluble antioxidant in animal cells, was considered as a major ROS scavenger which can block lipid peroxidation in cell membranes [69], therefore, it was predicted vitamin E could improve the development of porcine IVF embryos by suppressing cell membrane injuries caused by ROS [66]. Yuh et al.[70] have reported that the effect of vitamin E supplementation in NCSU-23 medium on porcine parthenogenetic embryos, it increased the average number of total cells at the blastocyst and decreased apoptotic cells at blastocyst as compared to control (without supplementation). In addition, it increases the proportion of porcine embryos reaching the blastocyst stage, reduces the average number of apoptotic nuclei and

DNA fragmentation nuclei [66] and the triglyceride content [71] in pig blastocyst after IVC. These results are agreement with previous reports the positive effects of vitamin E on the normal proportion and quality of cultured bovine blastocysts and on the accumulation of lipids in these blastocysts [72].

Recently, to facilitate nuclear reprogramming and thus improve cloning efficiency, several methods treating early nuclear transferred embryos with DNMT1 inhibitors like 5-aza-20-deoxycytidine (5-aza-dC) [73] and histone deacetylase inhibitors (HDACi) like TSA [74], scriptaid [75] and oxamflatin [76], have been tested to assist the somatic nucleus to mimic DNA methylation and chromatin remodeling. They found beneficial in improving cloning successful rate and correcting gene expression in pigs.

Due to many attempts to improve IVP of porcine embryos, now porcine embryos can now provide viable sources more efficiently with less cost and time compared with the *in vivo* counterparts.

#### 1.4. Reactive Oxygen Species

During a process of the energy generation in mitochondria, reactive oxygen species (ROS) are produced as product of the metabolism of oxygen in addition to energy. ROS are chemically reactive molecules containing oxygen. In normal situation, ROS have important roles in cell signaling and homeostasis [77]. However, during times of changeable environment like UV or heat exposure as well as conditions of internal and external oxidative stress, ROS levels can increase dramatically. This may result in significant damage to cell structure such DNA, RNA and proteins and contributes to the physiology of cellular senescence. Commonly, this is known as oxidative stress. Generally, oxidative stress results due to the loss of balance between ROS production and antioxidant defenses [78]. For example, oxidative damage resulted by  $\text{H}_2\text{O}_2$  can be ameliorated by catalase and superoxide dismutase by converting these compounds into oxygen and water, benign molecules. However, if this conversion is not totally efficient, residual peroxides will persist in the cell. So, excessive amounts of ROS can cause deleterious effects to DNA, RNA and protein in the cell [79].

ROS mainly include superoxide anion radical ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $^{\cdot}\text{OH}$ ), so on, concerned with process of reducing electrons gradually. These ROS are highly reactive to remove the electron from other molecules. In general, deleterious attacks from excess ROS may ultimately end in cell death and necrosis. These harmful attacks are mediated by the following more specialized mechanisms. First, opening of ion channels was started. And then lipid

peroxidation, oxidations of polyunsaturated fatty acids in lipids, occurred. Subsequently, oxidation of amino acid in proteins was worked. Finally, DNA oxidation was happened [80].

Current studies demonstrate that the accumulation of ROS can particularly decrease an organism's fitness in brain because oxidative damage is a contributor to senescence. In other words, the brain is particularly vulnerable to oxidative damage. Specially, due to the accumulation of oxidative damage, cognitive dysfunction is caused in rat experiment [81]. Accumulating oxidative damage can then affect the efficiency of mitochondria and further increase the rate of ROS production [82]. The accumulation of oxidative damage may be an etiology of human degenerative disease such as Alzheimer's disease. Many researchers accepted that oxidation of cellular proteins is potentially important for brain function [83].

Similarly, oxidative stress is caused in female reproductive system. This stress ratio can be altered by increased levels of ROS or a decrease in antioxidant defense mechanisms [80]. Excessive ROS production may hamper the body's natural antioxidant defense system of female, creating an environment unsuitable for normal physiological reactions. This, in turn, can lead to a number of reproductive disease including endometriosis, polycystic ovary syndrome, and unexplained infertility. It can also cause complications during pregnancy, such spontaneous abortion, recurrent pregnancy loss, preeclampsia, and intrauterine growth restriction. Moreover, in steroidogenic tissues such as the ovary, steroidogenic enzymes could be also sources of ROS [84].

Antioxidants are scavengers that detoxify excess ROS, which helps maintain the body's delicate oxidant/ antioxidant balance. There are two types of antioxidants : enzymatic and non-enzymatic. Enzymatic antioxidants possess a metallic center, which gives them the ability to take on different valences as they transfer electrons to balance molecules for the detoxification process. They neutralize excess ROS and prevent damage to cell structures. Endogenous antioxidants enzymes include SOD, catalase, glutathione peroxidase (GPx) and glutathione oxidase [85]. The non-enzymatic antioxidants consist of dietary supplements and synthetic antioxidants such as vitamin C, taurine, hypotaurine, vitamin E, Zn, selenium, beta-carotene and melatonin.

Also, ROS may originate from embryo metabolism and/or embryo surroundings. Embryo metabolism generates ROS via several enzymatic mechanisms. Generated ROS can induce development block and retardation [86]. Many antioxidants can alleviate oxidative stress during reproductive processes, and can enhance embryonic development *in vitro*. In pigs, as in other mammals, several antioxidants have been used as supplements in culture media to enhance embryonic development. These are as in the following; Ascorbic acid (vitamin C) [87], tocopherol (vitamin E) [88], selenium [89], insulin-transferrin-selenium (ITS) [90],  $\beta$ -carotene [91], retinoic acid (vitamin A) [92], lycopene [93], biochanin A [94] and anthocyanin [95]. Like this, many studies have continued its efforts that reduced ROS generated from metabolism by adding exogenous antioxidants during culture of embryo in pig.

## 2. OBJECTIVE

The purpose of this study is to investigate effects on porcine embryo as change of culture condition resulted from different oxygen tension and to increase *in vitro* production rates of porcine embryos by supplementing some antioxidants to the media for defending of oxygen toxicity. So this thesis is comprised of two part, one is to investigate the development competence of embryos during IVM and IVC on different oxygen tension, and the other is to investigate the effect of some antioxidants supplement into IVM media on the embryonic development. For the effect of different oxygen tension, porcine oocytes were matured in the two oxygen concentration (5% and 20%), and then cultured in the two oxygen concentration (5% and 20%), respectively (Part III). Each groups were checked *in vitro* developmental rate and multiple gene expression patterns. In next part, for the effects of antioxidant supplementation, porcine oocytes were matured in media with melatonin (part IV, chapter 1), quercetin (part IV, chapter 2) and taxifolin (part IV, chapter 3), respectively, and then cultured for 7 days after parthernogenetical activation. In this part, *in vitro* developmental rate, ROS and GSH level were checked. Lastly, final conclusion in this thesis was described in part V.

**PART II**

**GENERAL METHODOLOGY**



## **1. Chemicals**

Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

## **2. Oocyte Collection and *In vitro* Maturation**

Pig ovaries were collected from a local abattoir and transported to the laboratory in 0.9% (w/v) NaCl solution at 25-30°C. Follicular contents from antral follicles (3-6 mm in diameter) were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. The contents were pooled in a conical tube at 39°C and allowed to settle for a few minutes. The sediment was aspirated and diluted with Dulbecco's PBS (D-PBS; Invitrogen, USA) containing 100 U/mL penicillin G and 100 mg/mL streptomycin sulphate (pen-strep; Invitrogen, USA). Cumulus-oocyte complexes (COCs) with intact compact cumulus cell layers were selected and washed 3 times in TCM-Hepes before being transferred to a modified TCM-199 supplemented with 10 ng/ml EGF, 0.57 mM cystine, 0.91 mM sodium pyruvate, 5 µg/ml insulin, 1% (v/v) pen-strep, 0.5 µg/ ml follicle stimulating hormone, 0.5 µg/ ml luteinizing hormone and 10% porcine follicular fluid. For the first 22h only, the IVM medium also contained Gonadotropin. The COCs were cultured at 38°C with 5% CO<sub>2</sub> at maximum humidity. After 44 hr of maturation, oocytes were denuded of cumulus cells by pipetting with 0.1% hyaluronidase in D-PBS supplemented with 0.1% polyvinyl alcohol. Then denuded oocytes were treated according to each experimental design.

### **3. Assessment of meiotic maturation of matured oocytes**

The meiotic maturation was determined by evaluating for the presence of the polar body. After 44 hr of IVM, the denuded oocytes were fixed in methanol for 15 min, mounted on a slide and stained with Hoechst 33342 in D-PBS. The presence or absence of the polar body was determined under UV light.

### **4. Parthenogenetic activation of mature oocytes and *in vitro* culture**

At 44 hr of IVM, metaphase II oocytes were parthenogenetically activated. Briefly, denuded oocytes were equilibrated for 1 min in 0.26 M D-mannitol-based activation solution supplemented with 0.1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, and 0.5 mM HEPES. The oocytes were then transferred to a chamber between two electrodes spaced 3.2 mm apart and overlaid with activation solution. The oocytes were activated by electric stimulation with a single direct current pulse of 2.0 kV/cm for 60  $\mu$ sec using a BTX Electro-Cell Manipulator 2001 (BTX, USA). Parthenogenetically activated oocytes were cultured in 500  $\mu$ l porcine zygote medium-5 (Funakoshi, Japan) for 7 days at 39 °C in a humidified atmosphere with 5% CO<sub>2</sub> and 5% O<sub>2</sub>. The cleavage rate and blastocyst formation rate were checked at 48 and 168 hr of IVC, respectively.

In this study on embryonic development, I used parthenogenesis rather than *in vitro* fertilization or somatic cell nuclear transfer to assess oocyte competence for embryonic development. The parthenogenetic activation of oocytes can be used to evaluate the developmental competence of oocytes *in vitro* without confounding factors from spermatozoa [96] and a variety of other factors introduced during *in*

*vitro* procedures. Furthermore, parthenogenetic activation is relevant to cloning research, because artificial activation of an oocyte is an essential component of nuclear transfer protocols [97].

## **5. Assessment of embryo quality**

Blastocysts quality was assessed by Hoechst staining of the inner cell mass and trophectoderm cells according to standard procedures. Briefly, the blastocysts were washed in HEPES-buffered TALP medium and then incubated with TALP medium containing 25 µg/ml Hoechst stain for 15 min at 39 °C. The stained blastocysts were mounted onto glass slides under a cover slip and counted while examined with an inverted microscope (Nikon Corp., Japan) equipped to perform epifluorescence.

## **6. Measurement of intracellular GSH and ROS levels**

Oocytes were sampled after 44 hr of IVM and 2 d of IVC to determine intracellular GSH and ROS levels using CellTracker Blue CMF2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin) and the dichlorohydrofluorescein diacetate (DCHFDA) methods, respectively, previously described [95, 98] with slight modification. Briefly, 20 oocytes and embryos from each treatment group were incubated in the dark at 39°C for 30 min in TALP medium supplemented with 10 uM CellTracker and 10 uM DCHFDA. After incubation, oocytes were washed in HEPES-buffered TALP medium, placed on a glass slide and covered with a cover slip. The fluorescence emissions from the oocytes and embryos were recorded as TIFF files using a cooled CCD camera attached to a fluorescence

microscope (Axio Photo; Carl Zeiss Jena GmbH, Germany) with excitation filters (460nm for ROS and 370 nm for GSH). The recorded fluorescent images were analyzed using image J software 1.55 (National Institutes of Health, USA) by counting the number of pixels after color inversion [99].

## **PART III**

# **THE EFFECT OF DIFFERENT OXYGEN TENSION DURING EMBRYO CULTURE OF PORCINE OOCYTES *IN VITRO***

# **Chapter 1. Developmental competence of porcine oocytes after *in vitro* maturation and *in vitro* culture under different oxygen concentrations**

## **1. Introduction**

Pigs share many characteristics such as anatomy, physiology and body size with humans and are expected to become important animal models for therapeutic cloning and xenotransplantation [100, 101]. Because of this, IVM of oocytes and IVP of blastocysts in pigs are very important initial steps in the study of early embryonic development and the production of transgenic animals. Recently, the IVP of blastocysts in pigs was increased by technical improvements in several of the developmental stages [58]. However, the IVP rate is still inferior to *in vivo* embryo production, and the cloning efficiency after embryo transfer is still low [102]. To increase the rate of *in vitro* blastocyst formation and to improve cloning efficiency after embryo transfer, it is necessary to produce high-quality matured oocytes.

The oocyte and its surrounding cumulus cells in mammals are metabolically connected through gap junctions that serve as a unique means of entry into the ooplasm for several metabolites. Cumulus cells have a close connection with oocytes during the course of maturation. Gonadotropins, steroids and other factors

from the follicle cells also interact with oocytes to provide essential support for *in vivo* maturation of oocytes. It is generally accepted that cumulus cells support the maturation of oocytes to the metaphase II stage and are intimately involved in “cytoplasmic maturation” of oocytes, which is the capacity to undergo normal fertilization and subsequent embryonic development [103].

Generally, *in vitro* culture of porcine oocytes is maintained at higher concentrations of oxygen than the *in vivo* environment. Increased partial pressure of oxygen generates excessive amounts of cytotoxic ROS and may directly affect the viability of embryos [104]. Therefore, oxygen tension during culture would be an important factor affecting both oocyte maturation and embryo development. In cattle, several studies have shown that the efficiency of oocyte or embryo culture could be improved by decreasing oxygen concentration [105, 106], whereas others were contradictory [107, 108] or even showed that oxygen level had no significant effects on maturation and fertilization *in vitro* [109]. In mice, it was reported that a significant decrease in total cell number in blastocysts occurred as oxygen concentration increases during IVM [110]. In pigs, immature oocytes are commonly matured *in vitro* under a relatively high (20%) oxygen concentration although oxygen concentration is much lower than in the atmosphere both in the reproductive tract [111] and follicular fluid [112]. On the other hand, embryo culture is usually conducted *in vitro* under a relatively low (5%) oxygen concentration because fertilization occurs *in vivo* under low oxygen tension [113, 114]. In view of all these reports, it is clear that the effect of oxygen concentration during IVM or IVC is not yet fully understood.

The aim of this chapter was to observe porcine embryo development in terms of oocyte maturation and blastocyst formation derived from culture of these stages under different oxygen concentrations and to analyse the concomitant effects of oxygen on transcript abundance of genes related to metabolism, oxidative response, apoptosis and developmental competence in cumulus oocyte complexes and blastocysts.



## 2. Materials and methods

### 2.1 Oocyte collection and *in vitro* maturation

Detailed protocols were described in general methodology (Part II). After culture of 44 hr, the meiotic maturation was determined by evaluating for the presence of the polar body as described in general methodology. After each maturation period, oocytes were denuded from COCs by pipetting with 0.1% hyaluronidase in Dulbecco's PBS (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% polyvinyl alcohol (PVA-DPBS). Then denuded oocytes and separated cumulus cells were collected for total RNA extraction.

### 2.2 Parthenogenetic activation of matured oocytes and *in vitro* culture

*In vitro* culture of activated embryos was basically equal to that described as general methodology (Part II). Follows experimental design, a group of approximately 40 to 50 oocytes were cultured in 500  $\mu$ l PZM-3 supplemented with 4 mg/ml fatty acid-free BSA. Briefly, each maturation group was further subdivided into two groups for culture at 39 °C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% (C5) or 20% O<sub>2</sub> (C20), resulting in four groups (M5C5, M5C20, M20C5, M20C20). The cleavage rate and blastocyst formation rate were checked at 48 and 168 hr of IVC, respectively. Thus, the experimental design was a 2 x 2 factorial, with IVM and IVC x 5% and 20% oxygen. Blastocyst for RNA extraction and assessment of quality were randomly selected as equal number in each groups. The quality of blastocysts was assessed by Hoechst staining as general methodology.

### 2.3 Total RNA extraction, RT-PCR and Real Time PCR

Total RNA was isolated from fresh or previously frozen (-80°C) cumulus cells, oocytes matured either at atmospheric or low oxygen concentration (M20 or M5, respectively) and blastocysts obtained from oocytes matured at atmospheric oxygen concentration and cultured at atmospheric or low oxygen concentration (C20 or C5, respectively) using the easy-spin<sup>TM</sup> (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Korea) according to the manufacturer's instructions, and quantified by a spectrophotometer and immediately stored at -80°C until used for RT-PCR and qRT-PCR. cDNA (complementary DNA) was produced from 1µg of total RNA extracted from cumulus cells, oocytes and blastocyst samples, using a SuperScript<sup>TM</sup> III First-Strand cDNA Synthesis Kit (Invitrogen Life Technologies) primed with oligonucleotide-dT (18mer) and followed by RNase H digestion of RNA, in a total volume of 20 µl as per the manufacturer's instructions. Real Time PCR was done according to the TAKARA BIO INC. with little modification. In brief, all the primers were standardized by standard curve. The PCR plate (MicroAmp optical 96-well reaction plate, Singapore) was made by adding 2 µl cDNA, 1µl forward primer, 1 µl reverse primer, 10 µl SYBR Premix Ex Tag (Takara Bio Inc), 0.4 µl ROX Reference Dye (Takara Bio Inc.) and 5.6 µl of Nuclease-free water (Ambion Inc., Applied Biosystem). For each sample, four replications were made in a plate. The wells were capped by using MicroAmp optical 8-cap Strip, USA. The plate was then vortexed (Vortex-2, Genie, Scientific Industries Inc, Bohemia, N.Y. 11716, USA) and centrifuged briefly in a plate spinner (Plate Spin, Kubota). Real time PCR was done by using a 7300 Real Time PCR System (Applied Biosystems, Singapore)

according to the company instructions. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1 containing the specific primer to amplify glucose transport 1 (GLUT1), lactate dehydrogenase A (LDHA), glucose-6-phosphate dehydrogenase (G6PD), Mn-superoxide dismutase (MnSOD), glutathione peroxidase (GPX1), insulin-like growth factor receptor 2 (IGFR2), Bcl-2-associated X protein (BAX), B-cell lymphoma 2 (Bcl2), prostaglandin G/H synthase-2 (PTGS2, also known as COX2), the M-phase promoting factor subunit Cyclin B1 (CCNB1), Gremlin (GREM1), DNA-methyltransferase 3 beta (DNMT3B), aldo-keto reductase family 1 member B1 (AKR1B1), POU class 5 homeobox 1 (POU5F1) and caudal type homeobox 2 (CDX2).

## 2.4 Statistical analysis

Each experiment was repeated at least 4 times. All statistical analyses were performed using Prism4 software (GraphPad, U.S.A.). *t-test* was used for oocyte maturation and embryo development and Two-way analysis of variance (ANOVA) was used to determine significant differences in data levels followed by a Bonferroni post-tests to determine statistical differences of development rate and mRNA abundance among groups. Significant differences among the treatments were determined when  $P < 0.05$ . Data are expressed as means  $\pm$  S.E.M..

**Table 1.** Details of primers used for quantitative reverse transcription-polymerase chain reaction

Gene	Primer sequences (5' → 3')	Length of PCR product (bp)	Gene bank accession no. or References
<b>GLUT1</b>	F: 5'- GCTTCCAGTATGTGGAGCAACT R: 5'- AAGCAATCTCATCGAAGGTCC	132	X17058.1
<b>LDHA</b>	F: 5'- ATCTTGACCTATGTGGCTTGGA R: 5'- TCTTCAGGGAGACACCAGCAA	214	NM_001172363.1
<b>CCNB1</b>	F:5'- TTGACTGGCTAGTGCAGGTTC R:5'- CTGGAGGGTACATTTCTTCATA	368	NM_001170768.1
<b>Bcl2</b>	F:5'- TGGTGGTTGACTTTCTCTCC R:5'- ATTGATGGCACTAGGGGTTT	139	AF216205
<b>G6PD</b>	F: 5'- CCTCCTGCAGATGCTGTGTCT R: 5'- CGCCTGCACCTCTGAGATG	112	L.Jiang et al. [115]
<b>GPX1</b>	F: 5'- GATGCCACTGCCCTCATGA R: 5'- TCGAAGTTCCATGCGATGTC	80	AF532927
<b>IGFR2</b>	F: 5'- CGCTCTCTGCCTCTAGCAGT R: 5'- CCTACACCCCAAGTCTGGAA	225	AF342812
<b>MnSOD</b>	F: 5'- GCTTACAGATTGCTGCTTGT R: 5'- AAGGTAATAAGCATGCTCCC	101	S67818.1

<b>GREM1</b>	F: 5'- AACAGCCGTACCATCATCAAC R: 5'- TTCAGGACAGTTGAGAGTGACC	156	NM_001082450.1
<b>BAX</b>	F: 5'- GCCGAAATGTTTGCTGACGG R: 5'- CGAAGGAAGTCCAGCGTCCA	152	AJ606301
<b>PTGS2</b>	F: 5'- CTGCCGTGTCGCTCTGCACTG R: 5'- TCATAACTCCATATGGCTTGAAC	287	AY028583
<b>DNMT3B</b>	F: 5'- AGTGTGTGAGGAGTCCATTGCTGT R: 5'- GCTTCCGCCAATCACCAAGTCAAA	133	NM_001162404.1
<b>AKR1B1</b>	F: 5'- AAGGAGCACAGTTCCAAGCAGTCA R: 5'- CCCGAAGAGCACTACCTGTAGATT	166	CO994619
<b>POU5F1</b>	F: 5'- TTTGGGAAGGTGTTTCAGCCAAACG R: 5'- TCGGTTCTCGATACTTGTCCGCTT	198	NM_001113060
<b>CDX2</b>	F: 5'- TGTGCGAGTGGATGCGGAAG R: 5'- CCGAATGGTGATGTAGCGACTG	149	gi   262070767
<b><math>\beta</math>-actin</b>	F: 5'- GTGGACATCAGGAAGGACCTCTA R: 5'- ATGATCTTGATCTTCATGGTGCT	137	U07786

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### **3. Results**

#### **3.1 Effects of oxygen concentration on oocyte nuclear maturation**

A total of about 300 oocytes were used in eight replicates to evaluate the effects of 5% vs. 20% oxygen on nuclear maturation during IVM. The first polar body extrusion rate was not significantly difference between these two groups (Table 2).

**Table 2.** Effects of different oxygen concentrations during IVM on the frequency of first polar body extrusion

Concentration %	Oocytes, n	Oocytes with first polar body extrusion, n	First polar body extrusion rate (% $\pm$ S.E.M.)
<b>5</b>	310	258	83.22 $\pm$ 1.6
<b>20</b>	311	266	85.53 $\pm$ 0.8

First polar bodies were counted by Hoechst staining after 44 hr maturation *in vitro*.

No differences between groups were observed.

### 3.2 Effects of oxygen concentration on subsequent development of porcine oocytes *in vitro*

The number of blastocysts was significantly increased in the 5% IVC group vs. the 20% IVC group on further culture after maturation at atmospheric oxygen concentration. Moreover, the group matured under 20% oxygen and cultured under 5% oxygen (M20C5 group) displayed higher development than the other groups ( $P < 0.05$ ). No differences were observed among the M5C5, M5C20 and M20C20 groups. The oxygen concentration had no effect on the first cleavage frequency or the cell number per blastocyst (Table 3). Also, blastocyst morphology or degree of expansion was not different among the groups.



**Table 3.** Overall cleavage rate and blastocyst yield following maturation (M group) and further development in culture (C group) under atmospheric (20%) or low (5%) oxygen

Treatment	Embryos examined, n	Cleavage, n (%) <sup>*</sup> ≥2 cell	Blastocysts, n (% ± S.E.M.)	No. of cells per blastocyst (means ± S.E.M.)
<b>M5C5</b>	118	70 (59.32)	17 (14.41) <sup>a</sup>	34.33 ± 3.7
<b>M5C20</b>	117	72 (61.54)	12 (10.26) <sup>a</sup>	32.40 ± 1.6
<b>M20C5</b>	126	88 (69.84)	29 (23.02) <sup>b</sup>	31.83 ± 2.3
<b>M20C20</b>	123	85 (69.11)	22 (17.89) <sup>ab</sup>	33.20 ± 4.6

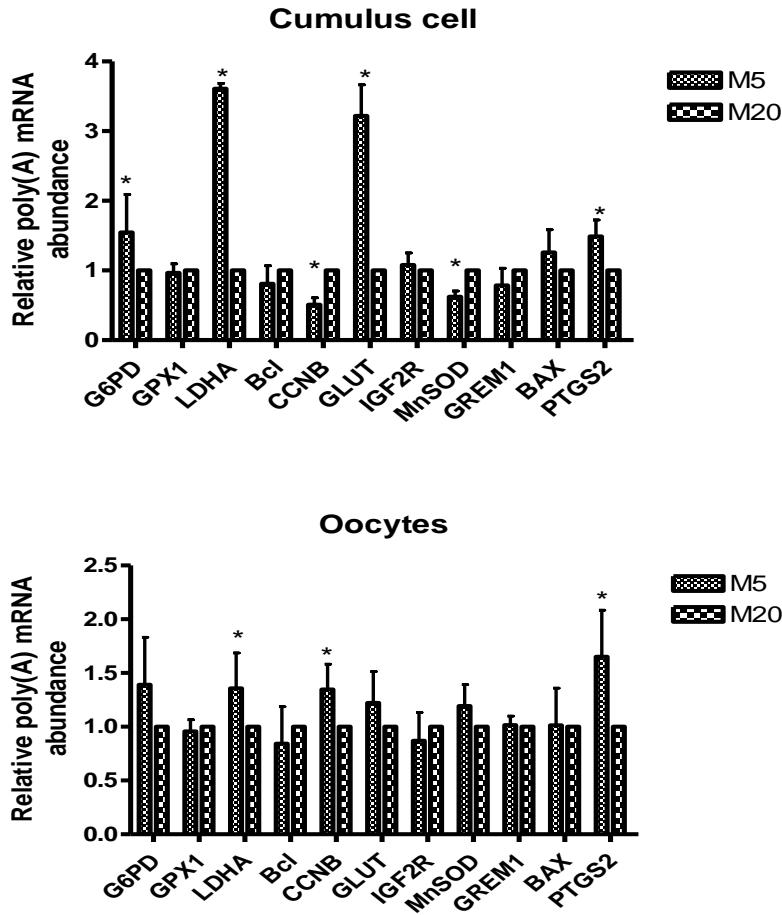
Percentages are based on the number of embryos examined.

<sup>a, b</sup> : Within the same column, values with different superscripts were different (P<0.05).

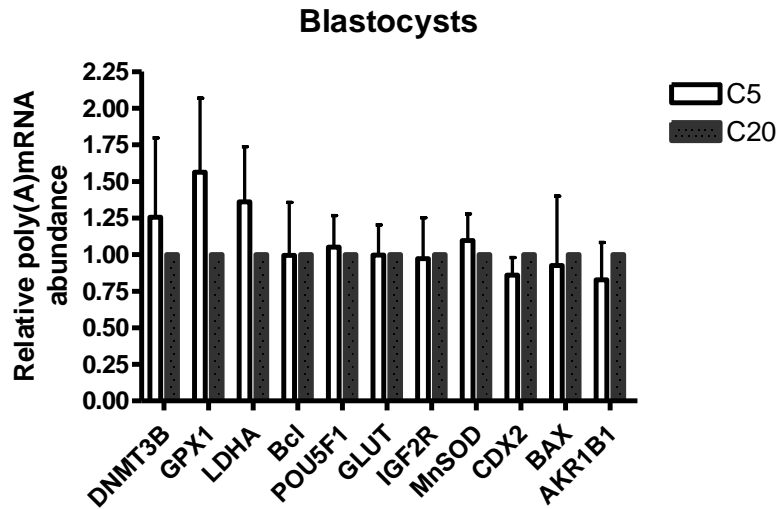
### 3.3 COC and blastocyst relative poly(A) mRNA abundance

The relative poly(A) mRNA abundance of eleven genes was analyzed in cumulus cells and oocytes, as shown in Figure 1. These genes were selected to analyze whether oxygen level affected biological functions essential to produce developmentally competent oocytes, such as glucose transport and metabolism, antioxidant response and apoptosis. Three of the genes analyzed are related to metabolism (GLUT1, LDHA and G6PD). GLUT1, LDHA and G6PD was up-regulated in cumulus cells matured in 5% oxygen compared with those matured under 20% oxygen ( $p<0.05$ ). Two of the genes analyzed are related to antioxidant response (MNSOD and GPX1). MnSOD was up-regulated in cumulus cells matured in 20% oxygen ( $p<0.05$ ). The growth factor IGFR2, proapoptotic gene BAX and anti-apoptotic gene BCL2 did not show significant differences among groups. Among genes selected for oocyte competence, the CCNB1 poly(A) mRNA content was significantly higher in cumulus cells matured under 20% oxygen, but the reverse was found in oocytes ( $p<0.05$ ). PTGS2 was up-regulated in cumulus cells and oocytes matured in 5% oxygen ( $p<0.05$ ). GREM poly (A) mRNA content showed no significant differences among groups.

In blastocysts resulted from parthenogenetically activated oocytes, I analyzed their quality in terms of transcription of additional genes (methylation : DNMT3B, blastocyst quality : AKR1B1, POU5F1 and CDX2) as well as the previously mentioned genes. No differences in mRNA abundance of these candidate genes were found among blastocysts derived from oocytes cultured under different oxygen concentrations (Figure 2).



**Figure 1.** Relative poly(A) mRNA abundance of 11 candidate genes related to metabolism (GLUT1, LDHA and G6PD), antioxidant response (MnSOD and GPX1), growth factors and apoptosis (IGF2R, Bcl 2 and BAX) and oocyte competence (CCNB1, PTGS2 and GREM1), in cumulus-oocyte complexes matured *in vitro* under 5% or 20% oxygen atmosphere. upper: cumulus cells, lower: oocytes. An asterisk (\*) indicates differences between groups based on one-way analysis of variance ( $P<0.05$ ).



**Figure 2.** Relative poly(A) mRNA abundance of 11 candidate genes related with metabolism (GLUT1 and LDHA), antioxidant response (MnSOD and GPX1), growth factors and apoptosis (IGF2R, Bcl2 and BAX), methylation (DNMT3B) and blastocyst quality (AKR1B1, POU5F1 and CDX2), in blastocysts produced from embryos cultured under 5% or 20% oxygen atmosphere. No differences were observed.

## 4. Discussion

The purpose of this study was to determine the relationship between different oxygen concentrations and maturation rate or developmental competence of porcine oocytes. Some studies of porcine *in vitro* maturation systems reported that low oxygen concentration during IVM or IVC has a beneficial effect [116, 117], but others indicated that is more effective for IVM or IVC [118] or even that oxygen level has no significant effects on IVM or IVC [109]. As a result, the optimal oxygen concentration is a controversial issue in porcine embryo production systems. In this chapter, I evaluated quantitative changes of some genes in embryos matured under different oxygen concentrations. Oxygen level during IVM had no significant effect on oocyte maturation. However, atmospheric oxygen concentration during IVM followed by IVC at 5% oxygen significantly improved embryo development. These results suggest that the use of an appropriate oxygen tension during IVM and IVC may be particularly useful in techniques that demand highly competent oocytes, such as ICSI, SCNT and oocyte vitrification.

To evaluate the effect of oxygen concentration, the relative mRNA abundance of some genes related to embryo quality was analyzed, comprising three metabolism-related genes (GLUT1, G6PD and LDHA), two genes related to antioxidant response (MnSOD and GPX1), two genes related to apoptosis (Bcl2 and Bax), one related to growth factor (IGF2R), and three genes related to oocyte competence (CCNB1, PTGS2 and GREM1).

The glucose transporter GLUT1, the enzyme involved in anaerobic glycolysis, LDHA, and the enzyme that catalyses the first and irreversible step of the pentose phosphate pathway, G6PD, are important for metabolism. It was found that the poly(A) mRNA abundance of GLUT1, LDHA and G6PD were higher in the cumulus cells of COCs matured under low oxygen, implying a higher glucose uptake and an increase in anaerobic glycolysis. Most of the ATP for oocyte maturation is provided by glycolysis or imported from granulosa cells. Energy substrates required for oocyte maturation may differ under low and high oxygen. Both glucose and glutamine, alone or in combination, can support preimplantation development of pig embryos [119]. Glucose is metabolized equally via aerobic and anaerobic pathways, although glycolysis becomes the dominant pathway as development progresses. From this information, it is evident that pig embryos have changing metabolic needs as they develop and undergo the accompanying environmental changes such as oxygen tension.

To test possible differences in ROS production, poly(A) mRNA abundance of two genes related to oxidative response in both cumulus cells and oocytes (MnSOD and GPX1) was analyzed. The results suggest differences between treatments in antioxidant response according to poly(A) mRNA abundance reflected in different oxygen levels, specifically being higher under 20% oxygen. However, no differences were found in relative abundance of the proapoptotic gene BAX poly(A) mRNA or the anti-apoptotic gene Bcl2 poly(A) mRNA in cumulus cells and oocytes, suggesting no change related to apoptosis under different oxygen concentrations. ROS can induce apoptotic cell death in oocytes cultured without

cumulus cells [104]. Cumulus cells may effectively protect oocytes against apoptosis caused by ROS generated by high oxygen concentration.

Finally, the relative poly(A) mRNA abundance of three genes related to oocyte competence (CCNB1, PTGS2 and GREM1) was analyzed. A higher poly(A) mRNA abundance of CCNB1 was observed in cumulus cells matured under high oxygen concentration and in the oocytes under low oxygen, which may suggest a higher activity of mitosis-promoting factor (MPF). CCNB1 is the principal molecule for regulation of mammalian oocyte maturation and synthesis of CCNB1 is necessary for GVBD induction in a normal time course. That is, oocyte is not required for the activation of MPF during the first meiosis, but that it is required for the second meiosis because of its promotion of CCNB1 accumulation [120, 121]. PTGS2 mRNA abundance in human cumulus cells was higher from oocytes that developed into higher quality embryos compared with lower quality embryos [122]. In a study using PTGS2 knockout mice, full cumulus expansion was not observed in ovulated COCs and *in vivo* fertilization was completely suppressed [123]. In porcine, the expression of PTGS2 mRNA were up-regulated by FSH and LH during maturation period [124] and it has also been correlated with oocyte competence improvement. In the present study, a higher expression level of PTGS2 mRNA was found in cumulus cells and oocytes from COC matured under low oxygen concentration.

Expression patterns of AKR1B1, POU5F1, CDX2 and DNMT3B relevant to early embryonic development provide information to assess the quality of

blastocysts derived from IVM/IVC [125, 126]. Not only was total cell number without significant differences in blastocysts, but also no differences were observed in mRNA abundance of these genes or of other previously mentioned genes associated with metabolism, apoptosis and antioxidant response. These results show that although blastocyst production rate is significantly different, different oxygen concentrations during IVC had no effect on the quality of blastocysts.

During follicular development, oocyte quality is affected by communication between the oocyte and surrounding cumulus cells. Removal of cumulus cells before IVM decreases the quality of oocytes in pigs [127]. Therefore, cumulus cells are considered to have an important role in oocyte maturation by regulating meiotic progression and by supporting cytoplasmic maturation. However, the mechanisms by which cumulus cells improve oocyte maturation are poorly understood. In this study, poly(A) mRNA abundance of multiple genes in cumulus cells varied depending on the oxygen concentration during IVM. This means that cumulus cells play their important roles during IVM selectively under different oxygen concentrations. Under low oxygen, cumulus cells increase glucose metabolism via anaerobic glycolysis, but under high oxygen the cumulus cells increase endogenous antioxidants as protection against ROS production. Because of these beneficial effects of cumulus cells, oocytes probably could be matured *in vitro* with no significant difference in maturation rate regardless of oxygen concentration. But, because embryos are cultured without cumulus cells during IVC, they lack this protection *in vitro* and thus may be susceptible to harmful effects of high oxygen.



In conclusion, the use of low oxygen concentration during IVC significantly improves embryo development in terms of numbers, but not blastocyst quality in terms of mRNA abundance of associated candidate genes.

## **PART IV**

# **THE EFFECT OF ANTIOXIDANTS ON MATURATION AND CULTURE OF PORCINE OOCYTES *IN VITRO***

# **Chapter 1. Effects of Melatonin on *in vitro* maturation of porcine oocyte and expression of Melatonin receptor RNA in cumulus and granulosa cells**

## **1. Introduction**

*In vitro* matured porcine oocytes have proven to be useful sources for assisted reproductive technologies such as IVF, ICSI, and SCNT. Due to their physiological similarity to human, the pig is a good model species for human disease. Moreover, transgenic cloned pigs can potentially be used for xenotransplantation. However, compared to *in vivo* derived embryos, the *in vitro* developmental porcine embryo is still low [128]. Therefore, the application of cloning technique will require an increase in efficiency through refinements *in vitro* oocyte maturation and embryo culture systems.

*In vivo*, oocytes and embryos produce endogenous ROS by various pathways [129]. *In vitro* handling and culture expose oocytes and embryos to oxidative stress resulting from events such as exposure to light, elevated oxygen concentrations, and disturbed concentrations of metabolites and substrates [130]. The ROS such as hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^-$ ) anions, or hydroxyl radical ( $OH^\cdot$ ), can damage cell membranes [131] and DNA [132] and may play a role in apoptosis

[98]. Therefore, it is important to protect oocytes against oxidative stress during *in vitro* maturation. One approach is to supplement the medium with antioxidant compounds.

Melatonin (N-aceyl-5-methoxytryptamine), an indole derivative secreted rhythmically from the pineal gland, plays a major role in regulating the circadian clock in mammals [133]. Also, this hormone has major effects on the reproductive system in mammals [134, 135]. More recent studies have demonstrated that, besides its multiple actions on different physiological processes, melatonin is an indirect antioxidant and a powerful direct scavenger of free radicals [136-139].

In contrast to the majority of other known radical scavengers, melatonin is multifunctional and universal [140-142]. It is soluble both in water and in lipids and hence acts as a hydrophilic and hydrophobic antioxidant. It has been shown that melatonin and its metabolites directly scavenge hydroxyl radical, organic oxyl radical, peroxy radical, peroxyxynitrite anion, nitric oxide and singlet oxygen [143-146]. Also, melatonin may repair some molecules that have been oxidized [147].

Melatonin has been successfully tested for promoting mouse embryo development *in vitro* [148]. It has been also reported as having no detrimental effects on mouse or rat embryo development during toxicity tests [149-151] performed either *in vitro* or *in vivo* [151]. Recently, an increased ratio of vitrified sheep blastocysts developing with melatonin during 24 and 48 hr *in vitro* post warming culture was reported [152].

Taken together, these data suggest that melatonin might have beneficial effects as an antioxidant agent during porcine oocyte maturation and/or embryo development. Besides the need to verify its previously described effects, it is not clear what stages of porcine pre-implantation development are influenced by melatonin. The present study examined the effects of melatonin on porcine oocyte maturation and pre-implantation development of embryos *in vitro*. The specific objectives were to evaluate 1) effects of melatonin supplementation during IVM on the frequency of extrusion of polar bodies and parthenogenetic development of embryos, 2) determine effects of melatonin on the ROS levels of *in vitro* matured cumulus oocyte complexes, 3) investigate the local expression of melatonin receptor I in cumulus cells, granulosa cells and oocytes.

## 2. Materials and methods

### 2.1 Experimental design

In order to determine effective concentrations for improving IVM of the oocyte (Experiment 1), melatonin was supplemented in each IVM medium at four concentrations (0, 10, 50 or 100 ng/ml) during the entire maturation period of 44 hr (the first half, eCG- and hCG-containing TCM-199 medium for 22 hr; and the latter half, eCG- and hCG-free TCM-199 medium for 22 hr). In experiment 2, we evaluated the effects supplementing the IVM medium with melatonin at the four concentrations on the parthenogenetic *in vitro* development of embryos. In experiment 3, I assessed the effects of similar melatonin concentrations in IVM medium on the ROS levels. Experiment 1 was designed to determine the presence of melatonin receptor on oocytes and granulosa and cumulus cells.

### 2.2 Oocyte Collection and *in vitro* Maturation

Detailed protocols were described in general methodology (Part II). But, TCM-199 supplemented with 0.1 % PVA was used instead of PFF as maturation media in this chapter. The meiotic maturation was determined by evaluating for the presence of the polar body as described in general methodology.

### 2.3 Activation of matured oocytes

For parthenogenetic activation, electrical activation protocol described in general methodology (Part II) was used.

#### 2.4 *In vitro* culture of activated parthenogenetic embryos

*In vitro* culture of activated embryos was basically equal to that described as general methodology (Part II). Follows experimental design, a group of approximately 40 to 50 oocytes were cultured in 500  $\mu\text{l}$  medium containing PZM-3 supplemented with 4 mg/ml fatty acid-free BSA. The quality of blastocysts was assessed by Hoechst staining as general methodology.

#### 2.5 Isolation of porcine granulosa and cumulus cells, RNA isolation and RT-PCR

Porcine granulosa cells were isolated as previously described [153, 154]. Follicular aspirates from ovary were centrifuged at 250 g for 10 min at 4 °C. The supernatant was then aspirated, and cell pellets from ovary were pooled and resuspended in PBS, overlaid onto a 60% (v/v) Percoll solution and centrifuged at 1000 g for 20 min at 4 °C. Granulosa cells precipitating at the Percoll-PBS interface were aspirated, resuspended in PBS and centrifuged at 250 g at 4 °C. This step was repeated three times. After the final wash, the supernatant was removed and the cell pellet was resuspended in PBS. Cell viability was determined by the trypan blue dye exclusion method.

Cumulus cells were removed from matured oocytes by pipetting in 0.1% hyaluronidase. Denuded oocytes and their respective cumulus cells were washed once in PBS, and total RNA was isolated from fresh or previously frozen (-80°C) granulosa cell, cumulus cells or fresh granulosa cells, cumulus cells, and oocytes using the RNeasy total RNA kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions, and quantified by a GeneQuant II spectrophotometer (Pharmacia, Uppsala, Sweden). The yield of total RNA was about 2~3  $\mu\text{g}$  RNA per

million granulosa cells, cumulus cells and oocytes. cDNA was produced from 5 µg of total RNA per porcine granulosa cells, cumulus cells and oocyte samples, using a SuperScript II reverse transcriptase kit (Gibco BRL, Grand Island, NY, USA) primed with oligonucleotide-dT (18 mer) and followed by RNase H digestion of RNA, in a total volume of 20 µl as per manufacturer's instructions. PCR was performed as described previously [155], using the following oligonucleotide primer. Porcine MT I primer sequence is positive strand 5'-tattgctacatctgacacagtc-3' and negative strand 5'-gccacaaacagccactctggga-3'. The PCR conditions were changed by reducing MgCl<sub>2</sub> concentration to 1.5 mM and the cycling conditions were 93°C, 3 min, followed by 35 cycles of 94°C, 30 sec; 64°C, 1 min; 72°C, 45 sec; plus a 5-min extension at 72°C. The product was a 461 bp fragment.

## 2.6 Measurement of ROS contents

Oocytes were sampled after IVM to determine intracellular ROS levels as previously described in general methodology of Part II.

## 2.7 Statistical analysis

All statistical analyses were performed using Prism4 software (GraphPad, U.S.A.). One-way ANOVA was used to determine significant differences in data levels. Tukey test was followed to determine statistical differences between groups. The significant differences among the treatments were determined when  $P < 0.05$ . Data are expressed as means  $\pm$  S.E.M..



### **3. Results**

#### **3.1 Effect of melatonin on porcine oocyte nuclear maturation**

A total of about 889 oocytes were used in five replicates to evaluate the effects of melatonin on nuclear maturation during IVM. The polar body extrusion rate was significantly higher ( $P < 0.05$ ) in the group supplemented with 10 ng/ml ( $84.6\% \pm 4.10$ ) when compared with the control group ( $75.6\% \pm 6.6$ ) and groups (Table 4).

**Table 4.** Effects of melatonin supplementation during IVM on the frequency of polar body extrusion

Concentrations (ng/ml)	Oocytes, n	Oocytes with polar body extrusion, n	Polar body extrusion rate (% $\pm$ S.E.M.)
0 (Control)	221	167	75.57 $\pm$ 2.94 <sup>a</sup>
10	227	192	84.58 $\pm$ 1.83 <sup>bc</sup>
50	220	178	80.91 $\pm$ 0.96 <sup>ab</sup>
100	221	169	76.47 $\pm$ 1.81 <sup>a</sup>

Polar bodies were counted by Hoechst staining after 44 hr maturation *in vitro*.

<sup>a,b,c</sup> Different letters indicate statistically significant differences ( $P < 0.05$ ).

### 3.2 Effects of melatonin on further development of porcine oocytes *in vitro*

A total of 672 oocytes that underwent IVM in media supplemented with four concentrations of melatonin were parthenogenetically activated in four replicates. Melatonin supplementation had no effect on the first cleavage frequency and the cell number per blastocyst (Table 5). However, significantly greater ( $P<0.05$ ) frequency of blastocysts developed in oocytes when IVM medium was supplemented with 50ng/ml melatonin. Supplementation of IVM medium with melatonin improved, at least numerically, the frequency of blastocyst development (Table 5).

**Table 5.** Effects of melatonin on further development of porcine oocytes *in vitro*

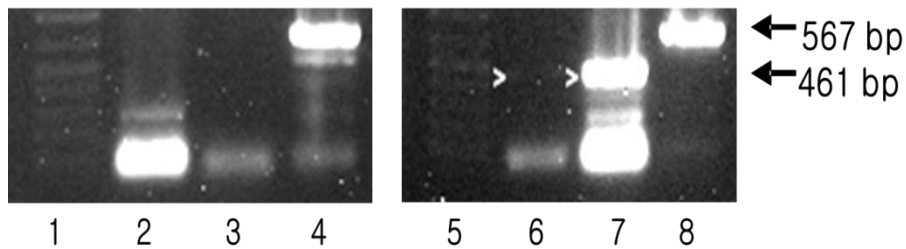
Concentrations (ng/ml)	Oocytes examined, n	Cleavage, n (%) <sup>*</sup> ≥2 cell	Blastocyst, n (% ± S.E.M.)	No. of cells per blastocyst (means ± S.E.M.)
0 (Control)	163	122 (74.85)	22 (13.28±2.101) <sup>a</sup>	60.50 ± 19.96
10	171	131 (76.61)	32 (18.50±2.377) <sup>ab</sup>	47.47 ± 13.63
50	175	134 (76.57)	38 (21.38±1.869) <sup>b</sup>	53.85 ± 16.84
100	163	124 (76.07)	34 (21.07±1.546) <sup>ab</sup>	55.87 ± 17.37

Percentages are based on the number of oocytes examined.

<sup>a, b</sup> : Within the same column, values with different superscripts were significantly different (P<0.05).

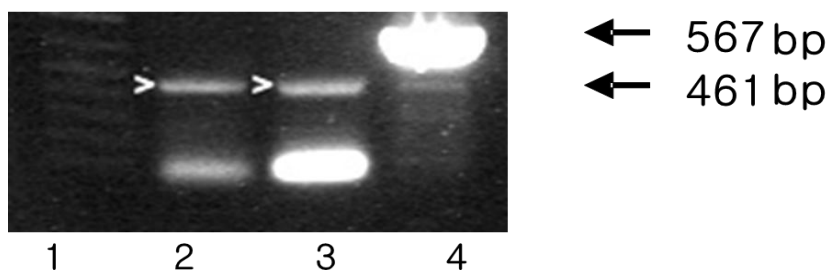
### 3.3 Expression of MT1 melatonin receptor mRNA expression in cumulus cells, granulosa cells and oocytes

MT1 melatonin receptor cDNAs were amplified from mRNA isolated from porcine cumulus cells (Fig 3, right) and granulosa cells (Fig 4) by RT-PCR with two rounds of amplification. PCR products obtained after the second round of amplification using nested primers were of the expected size (461 bp) for MT1 melatonin receptor. MT1 mRNA expression was not detected in porcine oocytes. (Fig. 3, left).



**Figure 3.** RT-PCR analysis of MT1 melatonin receptor mRNA expression in the porcine cumulus cells and oocytes

DNA gel electrophoresis of first (lane 2 and 6) or second (lane 3 and 7) amplification products generated from porcine cumulus cells or oocytes. Lanes 1 and 5, DNA molecular weight marker (DNA-*Hae*IV). Using MT1 nested primers, a band of 461 bp corresponding to the expected size for the amplified product of the MT1 melatonin receptor was obtained in cumulus cells (lane 6 and 7) but not in oocytes (lane 2 and 3) or  $\beta$ -actin primer (lanes 4 and 8).



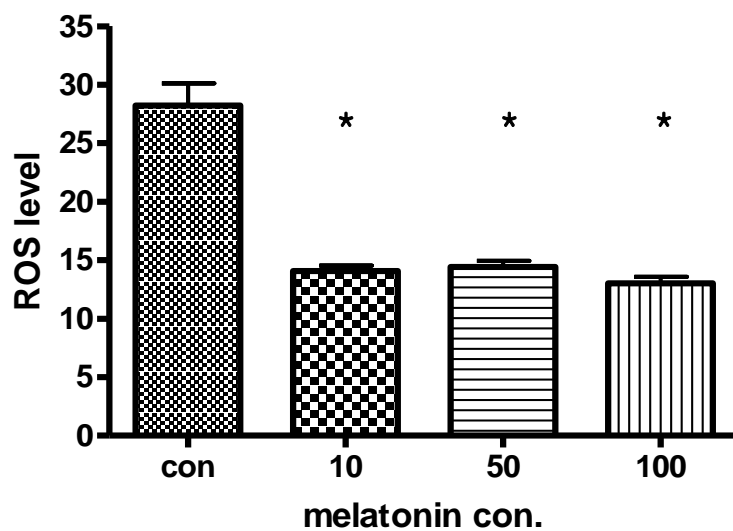
**Figure 4.** RT-PCR analysis of MT1 melatonin receptor mRNA expression in the porcine granulosa cells

DNA gel electrophoresis of first (lane 2) or second (lane 3) amplification products generated from porcine granulosa cells. Lane 1, DNA molecular weight marker (DNA-*Hae*IV). Using MT1 nested primers, a band of 461 bp corresponding to expected size for the amplified product of the MT1 melatonin receptor was obtained in granulosa cells (lane 2 and 3) or  $\beta$ -actin primer (lanes 4).

### 3.4 Measurement of ROS in porcine oocytes

In experiment 4, about 130 oocytes were used in four replicates to evaluate the effect of melatonin during IVM on the levels of ROS. ROS contents in oocytes matured in medium supplemented with melatonin, ranging from 13.0 to 14.4 pixels, was significantly lower in oocytes than those matured in control medium (28.2 pixels, Figure 5). Although the level of ROS was significantly reduced in melatonin-treated groups, there was no significant difference among any of the melatonin-treated groups.





**Figure 5.** Effects of four concentrations of melatonin on levels of ROS during IVM of porcine oocytes

The symbol (\*) indicates a significantly difference.

## 4. Discussion

The present study demonstrates that supplementing melatonin to IVM medium enhances oocyte maturation rate and embryo development. We found that melatonin improved the maturation rate of porcine oocytes during maturation in TCM-199. The rate of further development in blastocysts was also significantly higher when melatonin-treated oocytes were activated and further cultured in a PZM-3 medium. These findings demonstrate that melatonin supplementation of IVM medium improved both nuclear and cytoplasmic maturation.

My study on embryonic development was based on parthenogenesis rather than *in vitro* fertilization or somatic cell nuclear transfer to assess oocyte competence for embryonic development. The parthenogenetic activation of the oocytes can be used to evaluate the developmental competence of oocytes *in vitro* without confounding factors from the sperm [96] and a variable factors introduced during other procedures *in vitro*. Furthermore, parthenogenetic activation is relevant to cloning research, because artificial activation of an oocyte is an essential component of nuclear transfer protocols [156]. In mammals, parthenogenesis can yield viable offspring provided that parent-specific imprints regulating gene expression are overcome to permit the formation of a functional placenta [157]. Even without such manipulations, parthenogenesis still provides a valuable measure of oocyte competence to initiate the developmental program since development to the blastocyst stage is not affected by an epigenetic imprinting [158]. Accordingly, it is commonly used to assess oocyte competence to support

early development following somatic or pronuclear transfer [159, 160]. When compared to *in vitro* fertilization, the method of parthenogenetic activation used in my study has previously been shown to yield similar rates of development to the blastocyst stage [161], however, similar to my observation, these investigators recorded a significant reduction in cell numbers of the embryos resulting from parthenogenesis.

Recent studies have demonstrated that melatonin enhances *in vitro* embryo development in different species. According to Ishizuka et al.[148], melatonin at a  $10^{-6}$  M concentration supports fertilization and early embryo development after *in vitro* fertilization in mice. In cows, Papis et al. [162] reported that a relatively short period of *in vitro* culture with melatonin may have significant effects on the final development rate and, to some extent, on a quality (cell number) of bovine pre-implantation embryos fertilized *in vitro*. In addition, Rodriguez-Osorio et al. [163] reported that melatonin at a  $10^{-9}$  concentration has a positive effect on porcine embryo cleavage rates and blastocyst total cell numbers and it has a possible protective effect against heat stress.

In mammals, melatonin and its receptor (MT1 and MT2) mRNA are expressed in various tissues. Two mammalian melatonin receptor subtypes have been reported, MT1A and MT1B, both of which are G-protein coupled receptors [164]. The MT1A gene has been mapped in human, mice, sheep, pigs and cattle [155, 165]. The MT1B gene has been mapped to chromosome 9 in pigs [166]. However, the cellular expression and the role of melatonin systems have never been investigated

in porcine ovaries. Here, I report the presence of melatonin receptor (MT1) on cumulus cells and granulosa cells from porcine ovarian follicles.

Although melatonin is considered an exclusively neuronal hormone, its receptors have been found in tissues outside the nervous system. In particular, the binding of labeled melatonin and the presence of melatonin receptors has been documented in the human ovary [167]. Specific localization of MT1 melatonin receptors at the cellular level has only been demonstrated in granulosa cells [167]. The potential effect of melatonin on the differentiation of granulosa cells has not yet been established. The presence of high levels of melatonin in human preovulatory follicular fluid suggests that this hormone influences human ovarian and reproductive function [168, 169].

The present results show the presence of MT1 melatonin receptor transcripts in porcine cumulus cells and granulosa cells. The amount of transcripts seems to be lower compared with the amount of  $\beta$ -actin transcript. Presently, known mechanisms of melatonin action fall into three categories: receptor-mediated, protein-mediated and non-receptor-mediated effects [170]. Receptor-mediated melatonin events involve both membrane and nuclear binding site [171]. The effects of melatonin on reproductive function are thought to be mediated by G protein-coupled MT1 receptors in porcine. It is generally thought that melatonin influences reproductive functions at the level of the brain and pituitary. However, the presence of high levels of melatonin in follicular fluid [168] and its binding sites in the granulosa cells [172, 173] suggest that melatonin exerts its effects via a

receptor-mediated event at the level of the ovary. Some effects could involve modulation of steroidogenesis [174, 175] and luteolysis. The expression of receptor mRNA from cumulus cells and granulosa cells implies that melatonin may act directly on the oocyte for maturation.

As shown in Experiment 4, the present study also demonstrates that melatonin has antioxidative effects during *in vitro* maturation of oocytes. The beneficial effects of melatonin against oxidative stress and related damage in animals and humans, could improve mitochondrial function by counteracting mitochondrial oxidative stress [170].

The ROS may originate from embryo metabolism and/or embryo surroundings [130] and are detrimental to embryonic development [86, 176, 177]. The two-cell embryo block observed in mouse embryos was associated with a rise in ROS [86]. It has been suggested that the increase in ROS concentration during embryos cultured may lead to embryo apoptosis [161, 177]. Among ROS, H<sub>2</sub>O<sub>2</sub> plays a major role in apoptosis [178, 179].

Based upon results of the present study, I conclude that supplementation of IVM medium with melatonin could enhance porcine oocyte maturation and further embryonic development. Reduction of ROS could be an aspect of mechanism by which melatonin exerts its beneficial effects during oocyte maturation

## **Chapter 2. Quercetin improves *in vitro* development of porcine oocytes by decreasing reactive oxygen species levels**

### **1. Introduction**

Pigs have become useful animal models for organ xenotransplantation and as human disease models. Due to these important roles, *in vitro* culture of porcine oocytes is a very crucial process in the study of pre-implantation embryo development and in the production of transgenic animals. To increase the success rates of oocytes maturation and blastocyst formation *in vitro* and to improve cloning efficiency after embryo transfer, it is essential to produce high-quality matured oocytes. Many research studies have attempted to improve the production of high-quality oocytes by technical improvements across all stages of pre-implantation development. Among the most important factors influencing the developmental potential of embryos produced *in vitro* are the culture conditions including the external oxygen concentration that affect both oocyte maturation and embryo development [180].

ROS are endogenously produced by oocytes and embryos during *in vivo* development and IVC. Examples of ROS include oxygen ions and peroxides, particularly superoxide anions and hydroxyl radicals that are generated during the

process of oxygen reduction. Environmental stress can dramatically increase ROS levels. This may result in significant damage to cell membranes and plays a role in apoptosis. Additionally, the over-production of intracellular ROS in mammalian embryos during IVC is generally thought to be detrimental to embryo development [129]. Because pre-implantation embryos are particularly sensitive to ROS damage [130], the deleterious effect of ROS result in developmental inhibition [161]. In previous studies of pigs, IVF embryos with increased levels of ROS were found to have low developmental competence and increased DNA fragmentation [181]. Many reports have focused on overcoming these detrimental effects of ROS on embryo development. For example, reducing oxygen tension [116, 182] or treatment with antioxidants [183] during IVM or IVC improves embryo development. Therefore, it is important to protect oocytes against oxidative stress during IVM. One approach is to supplement the medium with antioxidant compounds during IVM.

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one) is a plant-derived flavonoid mainly found in fruits and vegetables. In mammals, flavonoids exert various biological and pharmacological effects [184]. Several studies have indicated that quercetin may have anti-inflammatory and antioxidant properties [185] due to its free radical scavenging and metal chelating properties [186]. In addition, this compound exerts a potent antioxidant effect. On the other hand, quercetin can also elicit pro-oxidant effects [187]. A relationship has been demonstrated between the free radical scavenging activity and anti-carcinogenic and anti-inflammatory properties of quercetin [188, 189].

Studies have been performed to evaluate the physiological functions and biological activities of quercetin in human and animal [190, 191]. However, there is limited information available on the effect of quercetin on oocyte maturation and embryonic development in pigs. Quercetin has been found to exhibit both estrogenic and anti-estrogenic effects in pigs *in vitro*, suggesting that this compound has different potential impacts on reproductive function [192].

Given this information, the objective of the present study was to examine the possible effect of quercetin supplementation during IVM and IVC on pig oocyte maturation and the developmental competence of parthenogenetically activated embryos. For this, I monitored the nuclear maturation and ROS levels of porcine oocytes, embryo cleavage and blastocyst formation of parthenogenetic embryos.



## 2. Materials and Methods

### 2.1 Experimental design

In order to identify the effective quercetin concentrations for improving oocyte maturation (Experiment 1), IVM medium was supplemented with four concentrations (0, 1, 10 or 50 µg/ml) of quercetin during the entire 44 hr maturation period. For experiment 2, I evaluated the effects of the same four concentrations of quercetin in IVM medium on the parthenogenetic development of embryos. In experiment 3, I assessed the effects these concentrations of quercetin in the IVM medium on the ROS levels in oocytes to assess the effect of antioxidant of quercetin.

### 2.2 Oocyte collection and *in vitro* maturation

Detailed protocols were described in general methodology (Part II). After culture of 44 hr, the meiotic maturation was determined by evaluating for the presence of the polar body as described in general methodology. After each maturation period, TALP medium was used as oocyte washing medium and denuding medium in this chapter.

### 2.3 Parthenogenetic activation of matured oocytes and *in vitro* culture

At 44 hr of IVM, metaphase II oocytes were parthenogenetically activated as described as general methodology (Part II). Following experimental design, A group of approximately 20 to 30 parthenogenetically activated oocytes were cultured in 500 µl PZM-5 for 7 days at 39 °C in a humidified atmosphere with 5%

CO<sub>2</sub> and 5% O<sub>2</sub>. The cleavage rate and blastocyst formation rate were checked at 48 and 168 hr of IVC, respectively. The quality of blastocysts was assessed by Hoechst staining as general methodology.

#### 2.4 Measurement of ROS level

Oocytes were sampled at 44 hr after IVM to determine intracellular ROS levels as previously described in general methodology (Part II).

#### 2.5 Statistical analysis

All statistical analyses were performed using Prism software (ver. 4.0; GraphPad, USA). A one-way ANOVA followed by a Tukey test was used to measure statistical differences among groups. *p*-values < 0.05 were considered to be statistically significant. Data are expressed as the means ± S.E.M..

### **3. Results**

#### **3.1 Effect of quercetin on porcine oocyte nuclear maturation**

About 782 oocytes were used for five replicate trials to evaluate the effects of quercetin on nuclear maturation during IVM. The polar body extrusion rate was not significantly different among the controls and groups treated with 1 or 10 ug/ml quercetin. In contrast, this rate was significantly lower ( $p < 0.05$ ) for the oocytes given 50 ug/ml quercetin ( $50.7\% \pm 1.9$ ) compared to the control ( $81.23\% \pm 1.0$ ) and other groups (Table 6).

**Table 6.** Effects of quercetin supplementation during IVM on the frequency of polar body extrusion

Concentration (ug/ml)	Oocytes, n	Oocytes with polar body extrusion, n	Polar body extrusion rate (% $\pm$ S.E.M.)
0 (Control)	192	156	81.23 $\pm$ 1.0 <sup>a</sup>
1	202	171	84.32 $\pm$ 2.4 <sup>a</sup>
10	197	160	80.97 $\pm$ 2.5 <sup>a</sup>
50	191	96	50.70 $\pm$ 1.9 <sup>b</sup>

Polar bodies were counted by Hoechst staining after 44 hr maturation *in vitro*.

<sup>a, b</sup> : Within the same column, values with different superscripts were significantly different (P<0.05).

### 3.2 Effects of quercetin on subsequent development of porcine oocytes *in vitro*

A total of 672 oocytes underwent IVM in media supplemented with four concentrations of quercetin. The oocytes were parthenogenetically activated and *in vitro* development was evaluated. Quercetin supplementation had no effect on the first cleavage frequency or cell number per blastocyst (Table 7). However, a significantly greater ( $p<0.05$ ) proportion of blastocysts developed into oocytes when the IVM medium was supplemented with 1 ug/ml quercetin. Addition of 1 ug/ml quercetin to the IVM medium improved the frequency of blastocyst development, but both the cleavage frequency and the rate of blastocyst formation of oocytes treated with the highest concentration of quercetin (50ug/ml) were significantly depressed compared to all other groups (Table 7).

**Table 7.** Effects of quercetin treatment of porcine oocytes on subsequent development *in vitro*

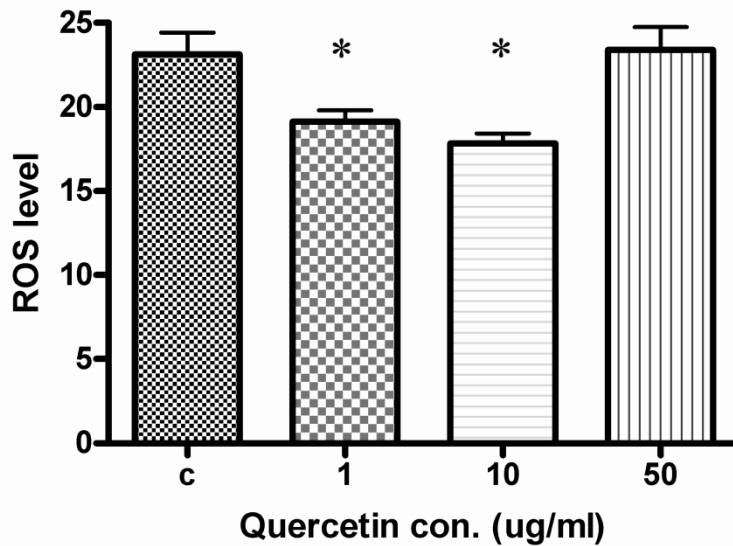
Concentration (ug/ml)	Oocytes examined, n	Cleavage, n (%) <sup>*</sup> ≥ 2 cell	Blastocysts, n (% ± S.E.M.)	No. of cells per blastocyst (means ± S.E.M.)
0 (Control)	191	123 (64.69)	18 (9.82±2.2) <sup>a</sup>	31.80 ± 2.6
1	171	135 (65.58)	32 (15.81±1.7) <sup>b</sup>	34.10 ± 2.0
10	160	105 (54.71)	17 (8.46±1.8) <sup>a</sup>	35.00 ± 3.0
50	96	48 (33.99) <sup>a</sup>	5 (3.46±1.6) <sup>c</sup>	31.40 ± 1.4

Percentages are based on the number of oocytes examined.

<sup>a, b, c</sup> : Within the same column, values with different superscripts were significantly different (P<0.05).

### 3.3 Measurement of ROS in porcine oocytes

For experiment 3, about 230 oocytes were used in four replicate trials to evaluate the effect of quercetin on the levels of ROS during IVM. The relative H<sub>2</sub>O<sub>2</sub> contents of oocytes matured in medium supplemented with 1 or 10 ug/ml quercetin ranged from 13.0 to 14.4 pixels, and were significantly lower than those in oocytes matured in control medium (Fig.6; 28.2 pixels,  $p<0.05$ ). However, the relative H<sub>2</sub>O<sub>2</sub> contents were not significantly different between the control and oocytes treated with 50 ug/ml quercetin.



**Figure 6.** Effects of four concentrations of quercetin on levels of ROS during IVM of porcine oocytes

The oocytes were stained with DCHFDA following maturation. Fluorescence intensity representing the concentrations of ROS generated in the mature oocytes was recorded using a cooled CCD camera attached to a fluorescence microscope. DCHFDA staining intensity was normalized to each imaged oocytes area (pixels).

\* indicates a significantly difference ( $p < 0.05$ )



## 4. Discussion

In the present study, the effects of quercetin as an antioxidant during IVM and IVC on oocyte maturation and embryonic development after parthenogenetic activation were examined, and the intracellular levels of ROS were measured. My results demonstrate that quercetin enhances the *in vitro* development of porcine oocytes. In quercetin-treated oocytes, ROS concentrations were significantly lower after maturation. Additionally, the rate of blastocyst formation was significantly higher in quercetin-treated oocytes that were activated and further cultured compared to the control group. On the other hand, excessive doses of quercetin were detrimental to oocytes and embryos without reducing the levels of ROS.

In recent years, interest in quercetin has gradually increased along with other flavonoids due to its health promoting activities that likely result from antioxidant effects in humans and animals. Nevertheless its overall biological impact of quercetin remains controversial, mostly due to the limited information about its bioavailability, endogenous dynamics and relative contribution of different types of conjugates. Some studies have showed that quercetin can mediate cancer cell apoptosis [193, 194]. Furthermore, these investigations have indicated that quercetin can selectively induce apoptosis of cancer cells and not normal cells. Other research has shown that quercetin can protect against oxidative stress by decreasing ROS generation through its antioxidant activity in normal human cells [195, 196]. My study demonstrates that quercetin at the optimal concentration acts

as an antioxidant and positively affects the maturation of porcine oocytes and *in vitro* embryonic development.

ROS are generated by embryonic metabolism or in the surrounding environment during IVC [197], and are detrimental to embryonic development [177]. Increased ROS levels are associated with the two-cell embryo block in mice [86]. It has been suggested that the increased ROS concentrations may lead to apoptosis during embryo culture [161]. Among different ROS, H<sub>2</sub>O<sub>2</sub> at high concentrations induces apoptosis [178]. Therefore, this study was conducted to monitor the level of H<sub>2</sub>O<sub>2</sub> within oocytes to indirectly assess ROS toxicity. I also examined parthenogenetic embryonic development to evaluate oocyte competence rather than *in vitro* fertilization or somatic cell nuclear transfer. The reason for this was because parthenogenetic activation can be used to evaluate oocyte developmental competence *in vitro* without confounding factors from sperm and a variety of other reagents introduced during *in vitro* procedure. Furthermore, parthenogenetic activation is relevant to cloning research because artificial oocyte activation is an essential component of nuclear transfer protocols [197].

The concentrations of quercetin used in this study (1, 10 and 50ug/ml) were selected based on previous results from a study of porcine granulosa cells [192]. In this study, 50ug/ml quercetin was found to inhibit progesterone production, modifies estradiol-17 $\beta$  production, and interferes with angiogenesis in granulosa cells by inhibiting vascular endothelial growth factor production, implying that quercetin may have a negative influence on ovarian physiology. Despite the

detrimental effect of quercetin on embryos, my study showed that treatment with adequate concentrations of quercetin (1 and 10 ug/ml) improved embryonic development, but it was not clear whether quercetin directly affects embryo development by decreasing ROS toxicity. I also found that rates of oocyte maturation and blastocyst formation were substantially reduced with a high concentration of quercetin (50ug/ml), although the expansion and total cell number of blastocysts were not adversely affected, demonstrating that quercetin has dose-specific effects on oocytes.

I suggest that the reduction of oocyte maturation and blastocyst formation rates with this concentration of quercetin may result from unresponsive signaling to oocytes and embryos or direct embryo toxicity owing to excessive levels of flavonoids. Some groups have reported toxic effects of other flavonoids on embryos from different species such as genistein [198], puerarin [199], ginkgolides [200] and purple sweet potato anthocyanins [201]. In contrast, several investigations have shown that supplementation of porcine IVM medium with antioxidants such as selenium, vitamin E and ascorbic acid decreases ROS levels while enhancing the developmental competence of IVF embryos and parthenotes [89, 181, 202]. Another study recently demonstrated that treatment with anthocyanin, a type of flavonoid, in IVM media improves the developmental competence of cloned pig embryos, most likely by increasing glutathione synthesis and reducing ROS levels [95]. Compared to a previous study conducted with other antioxidants, the effects of quercetin on the maturation rate, blastocyst formation rate and ROS generation rate in oocytes I observed were less potent.

In my investigation, no beneficial effect of quercetin treatment on the first polar body extrusion rate during IVM was found even though quercetin effectively reduced ROS levels. However, a beneficial effect of quercetin treatment at less than 10ug/ml was observed during subsequent culturing to the blastocyst stage. Therefore, it may be that the antioxidant effect of quercetin on oocytes was maximized during subsequent IVC, and thus, as suggested by previous studies and the present investigation, ROS may play a pivotal role in regulating oocyte maturation and embryonic development.

In conclusion, treatment of porcine oocytes with quercetin had a significant effect on embryonic development. At low levels, quercetin reduced intracellular ROS levels but was detrimental at high concentration. It is not clear whether this low level of quercetin is optimal in pigs. In addition, the rate of blastocyst formation was significantly increased by quercetin treatment whereas there was no increase in the number of blastocyst cells. Further studies are needed to determine the optimal concentration of quercetin and to ascertain the beneficial effects of this compound on pig embryo development.

# **Chapter 3. Effect of antioxidant flavonoids (Quercetin and Taxifolin) on *in vitro* maturation of porcine oocytes**

## **1. Introduction**

Increasing the efficiency of systems for *in vitro* production of porcine embryos is very important because pigs have high biomedical value for areas such as xenotransplantation and as models for stem cell research [203, 204]. However, despite intensive efforts, the yield and quality of IVM oocytes and embryos derived from them are still low compared with *in vivo* produced embryos. Improvements can be made by altering the culture conditions for oocyte maturation and embryo development, including the external oxygen concentration [180].

Oxidative stress originating from high external oxygen concentration can produce ROS [177], which may be responsible for damaging embryos and inducing early embryonic developmental blocks [130]. Therefore, supplementing maturation and culture media with antioxidants such as  $\beta$ -mercaptoethanol, cysteine and cysteamine can help to protect against defective embryo development [205, 206] . The glutathione (GSH) is intracellular free thiol compound, involved in protecting cell from ROS toxicity and regulates the intracellular redox balance. So, intracellular increase of GSH during *in vitro* process has beneficial effects on

porcine embryo development [36]. Also, supplementation of some antioxidants into IVM media could stimulate the synthesis of intracellular GSH, then improve embryo development in pig [205].

Flavonoids are a class of plant secondary metabolites and are most commonly known for their antioxidant activity *in vitro* [207]. Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one) and Taxifolin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one) are plant-derived flavonoids mainly found in fruits and vegetables. It has been reported that quercetin and taxifolin have anti-oxidative, anti-mutagenic and anti-inflammatory activities [186, 189, 208, 209] due to free radical scavenging. Also, Taxifolin is not mutagenic and has low toxicity compared to the related compound quercetin [210]. In previous chapter, I demonstrated that exogenous quercetin is beneficial for nuclear maturation during IVM and subsequent embryo development by reducing ROS levels [211].

However, little information is available on the effect of flavonoids on oocyte maturation and embryonic development in pigs. The objective of this chapter was to examine the effect of quercetin and taxifolin treatment during IVM and IVC on oocyte maturation and the development of parthenogenetically activated (PA) embryos. To this end, I observed nuclear maturation of oocytes, embryo cleavage and blastocyst formation of PA embryos, as well as intracellular levels of GSH and ROS in pig oocytes and embryos.

## 2. Materials and Methods

### 2.1 Experimental design

In order to determine effective concentrations of taxifolin for improving oocyte maturation (Experiment 1), it was included in the IVM medium (TCM-199) at four concentrations (0, 1, 10 or 50 µg/ml) during the entire maturation culture period of 44 hr. In experiment 2, I evaluated the effects of including quercetin or taxifolin in the IVM medium compared with a non-treated control group on the parthenogenetic development of embryos. In experiment 3, I assessed the effects of these quercetin and taxifolin concentrations in the IVM medium on the ROS and GSH levels in oocytes and embryos. Also, the effects of quercetin and taxifolin on maturation as phytoestrogen were assessed thorough hormone (P4 and E2) radioimmunoassay (experiment 4).

### 2.2 Oocyte collection and *in vitro* maturation

Detailed protocols were described in general methodology (Part II). After culture of 44hr, the meiotic maturation was determined by evaluating for the presence of the polar body as described in general methodology. After each maturation period, TALP medium was used as oocyte washing medium and denuding medium in this chapter.

### 2.3 Parthenogenetic activation of matured oocytes and *in vitro* culture

At 44 h of IVM, metaphase II oocytes were parthenogenetically activated as described as general methodology (Part II). Following experimental design, a

group of approximately 20 to 30 parthenogenetically activated oocytes were cultured in 500 µl PZM-5 for 7 days. The cleavage rate, blastocyst formation rate and the quality of blastocysts was checked as described in general methodology.

#### 2.4 Measurement of intracellular GSH and ROS levels

Oocytes were sampled after 44 hr of IVM and 2 d of IVC to determine intracellular GSH and ROS levels using the DCHFDA and CellTracker Blue CMF2HC methods previously described as general methodology.

#### 2.5 Effect of flavonoid on cumulus cell steroidogenesis

IVM media of both the first and second day of culture were collected, centrifuged at 1500 rpm for 3 min and the supernatants were stored at -20 °C until assayed for progesterone (P4) and estradiol-17β (E2) by validated radioimmunoassay. All samples were analyzed with assistance of the Neodin Veterinary Laboratory (Seoul, Republic of Korea; <http://www.vetlab.co.kr>).

#### 2.6 Statistical analysis

All statistical analyses were performed using Prism4 software (GraphPad, USA). One-way ANOVA was used to determine significant differences in the data followed by a Tukey test to determine statistical differences among groups. Significant differences among the treatments were determined when  $P < 0.05$ . Data are expressed as means ± S.E.M..



### **3. Results**

#### **3.1 Effect of taxifolin on porcine oocyte nuclear maturation**

A total of 746 oocytes were used in five replicates to evaluate the effects of taxifolin on nuclear maturation during IVM. The polar body extrusion rate was not significantly different among the control and the treatment groups at 1 or 10 ug/ml, but it was significantly lower ( $P < 0.05$ ) in the group containing 50ug/ml taxifolin ( $59.2\% \pm 7.9$ ) compared with the control ( $80.5\% \pm 3.5$ ) and the other treatment groups (Table 8).

**Table 8.** Effects of taxifolin during oocyte IVM on the frequency of first polar body extrusion (nuclear maturation)

Concentration (ug/ml)	Oocytes, n	Oocytes with polar body extrusion, n	Polar body extrusion rate (% $\pm$ S.E.M.)
0 (Control)	181	146	80.54 $\pm$ 3.5 <sup>a</sup>
1	192	162	84.43 $\pm$ 2.6 <sup>a</sup>
10	194	152	78.50 $\pm$ 3.2 <sup>a</sup>
50	179	107	59.21 $\pm$ 7.9 <sup>b</sup>

Polar bodies were counted by Hoechst staining after 44 hr maturation *in vitro*.

<sup>a, b</sup> Within a column, values with different superscripts are significantly different (P<0.05).

### 3.2 Effects of quercetin and taxifolin on PA embryo development

Based on the results from Experiment 1 and my previous study [211], 1 ug/ml of quercetin or taxifolin were used in Experiment 2. A total of 800 oocytes that underwent IVM in media supplemented with quercetin or taxifolin were parthenogenetically activated in nine replicates. Quercetin or taxifolin had no effect on the first cleavage frequency or the cell number per blastocyst (Table 9). However, a significantly greater ( $P<0.05$ ) proportion of blastocysts developed from oocytes when the IVM medium was supplemented with 1 ug/ml quercetin (Table 9). Further treatment with quercetin or taxifolin during IVC did not have any stimulatory effect on embryonic development (data not shown).

**Table 9.** Effects of quercetin treatment of porcine oocytes on subsequent development *in vitro*

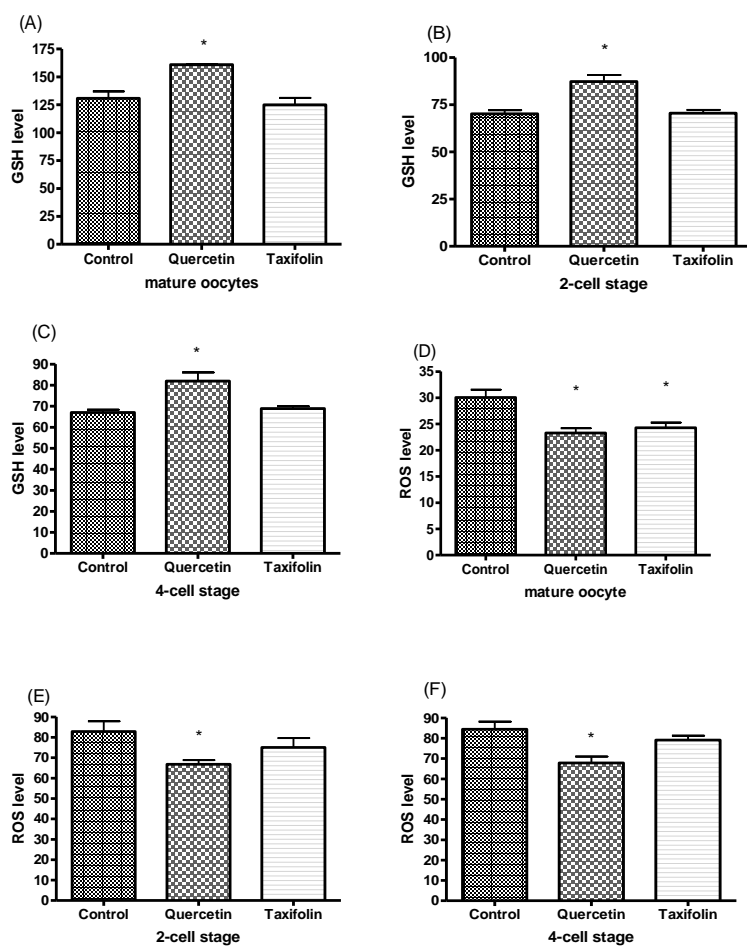
Treatment (1 ug/ml)	Oocytes examined, n	Cleavage, n (%) <sup>*</sup> ≥2 cell	Blastocysts, n (% ± S.E.M.)	No. of cells per blastocyst (means ± S.E.M.)
Control	265	182 (68.80)	44 (16.84±2.0) <sup>a</sup>	49.75 ± 3.7
Quercetin	266	188 (70.87)	64 (24.30±2.3) <sup>b</sup>	48.00 ± 2.6
Taxifolin	269	198 (73.58)	47 (17.74±1.9) <sup>a</sup>	51.86 ± 4.5

Percentages are based on the number of oocytes examined.

<sup>a, b</sup>Within a column, values with different superscripts are significantly different (P<0.05).

### 3.3 Intracellular levels of ROS and GSH in porcine oocytes and embryos

In Experiment 3, 430 oocytes were used in four replicates to evaluate the effect of quercetin and taxifolin during IVM and IVC on the levels of ROS and GSH. Quercetin increased intracellular GSH levels and decreased ROS generation in matured oocytes and in cultured embryos ( $p < 0.05$ ). Taxifolin also reduced the ROS levels in matured oocytes ( $p < 0.05$ ) but not in cleavage stage embryos, while GSH levels were not significantly different in oocytes or embryos compared with the control group (Figures 7).



**Figure 7.** Measurement of intracellular GSH and ROS levels in IVM oocytes and parthenogenetically activated embryos. The symbol (\*) indicates a significant difference ( $P < 0.05$ ). Values shown on the Y-axis are number of pixels.

### **3.4 Effect of flavonoid on cumulus cell steroidogenesis**

Basal steroid production by cumulus cells after 22 and 44 hr of culture is shown in Table 10. Progesterone and estradiol levels in culture media of oocytes incubated with and without flavonoid (QT or TF) were investigated. Both concentration of flavonoid (QT and TF) didn't significantly have differences.

**Table 10.** Effects of Flavonoid (QT or TF) on cumulus cell steroidogenesis

treatment	Estradiol (pg/mL)		Progesterone (ng/ml)	
	22hr	44hr	22hr	44hr
Control	1326.5	1161.7	85.0	323.7
Quercetin	1206.3	1144.3	80.7	295.3
Taxifolin	1223.8	1168.0	75.8	395.2

E2 and P4 production by cumulus cells after 22 and 44hr of *in vitro* maturation of oocytes in absence (control) or in presence of 1 ug/ml quercetin or 1 ug/ml taxifolin. Data represent mean of at least four replicates.



## 4. Discussion

In this study, effects of the antioxidants (quercetin and taxifolin) were examined during oocyte maturation and embryonic development following parthenogenetic activation, and on intracellular levels of ROS and GSH. Although porcine PA embryos cannot develop beyond 29 days of gestation *in vivo* [212], they may be good models to evaluate effects of exogenous factors during *in vitro* embryonic development. My results showed that the rate of PA blastocyst formation from quercetin-treated oocytes was significantly higher than in the control group and in the taxifolin-treated group. However, quercetin did improve the cleavage rate or total cell number of blastocysts compared with the other groups. Supplementing the IVM medium with taxifolin did not improve nuclear maturation, but was effective in reducing ROS levels in matured oocytes. The inclusion of quercetin but not taxifolin in the IVM medium increased PA blastocyst formation, presumably because quercetin reduced ROS and increased intracellular GSH more effectively than the taxifolin treatment. However, I have found no beneficial effect of quercetin or taxifolin treatment on blastocyst formation when applied only during IVC (data not shown). It is not clear whether improved embryonic development observed after treatment of oocytes with quercetin or taxifolin was due to a direct action of quercetin or taxifolin on embryos or to the reduction of ROS toxicity by increasing GSH.

ROS may originate from embryo metabolism and/or the embryo environment, and are detrimental to embryonic development [176, 177]. Many antioxidants can

alleviate oxidative stress during reproductive processes, and can enhance embryonic development *in vitro*. In pigs, as in other mammals, several antioxidants have been used as supplements in culture media to enhance embryonic development [197, 213], but little research has been done with flavonoids, which are well-known and powerful natural antioxidants. To the best of our knowledge, this is the first study investigating quercetin or taxifolin effects on early embryonic development *in vitro* in pigs. My data indicate that the optimal amount of flavonoid is concentration-specific, and while lower concentrations elicit no observable responses, excessive levels could be toxic to the oocyte.

Due to their structural similarity with estrogen, several flavonoids, including genistein and daidzein, also interact with the estrogen receptor to mediate their activity and thereby act as weak or moderate phytoestrogens [214, 215]. Female mice treated neonatally with genistein showed multi-oocyte follicles, lacked regular estrous cyclicity and showed implantation failure, although ovulated oocytes were developmentally competent [216]. Therefore, to confirm treatment effects of flavonoids as phytoestrogens, I assayed for P4 and E2 by radioimmunoassay during the oocyte maturation period (44hr). For P4 and E2, steroid concentrations are expressed steadily without differences among groups (control, 1 $\mu$ g/ml quercetin- and taxifolin- treated group) (Table 3). Maybe it thought that quercetin and taxifolin in this concentration is not effective in changing basal estradiol-17 $\beta$  secretion as phytoestrogens, and this finding is in agreement with the results of other flavonoids obtained by Galeati et al [214] in

porcine granulosa cells, showing that these flavonoids in this concentration may affect very weakly on oocytes or granulosa cells as phytoestrogens.

Previous studies reported that addition of high concentrations of antioxidants to the IVM medium decreased the rate of blastocyst formation compared to treatment with low concentrations [36, 217], suggesting that the proper concentration of an antioxidant can contribute to the generation of high quality embryos. In my experiments, treatment with 50ug/ml taxifolin was detrimental to oocyte maturation, which is consistent with the result using quercetin in my previous study [211]. One study reported that quercetin at 50ug/ml inhibited progesterone production by granulosa cells, altered estradiol-17 $\beta$  production, and interfered with angiogenesis by inhibiting VEGF production, so quercetin may have a negative influence on ovarian physiology [192]. Both quercetin and taxifolin in high concentration (at least 50ug/ml) may have detrimental effects to oocytes by influencing culture environments.

I compared effects of quercetin and its analogue taxifolin, both at 1 ug/ml, on embryo development after parthenogenetic activation (Experiment 2). Previous research has shown that using antioxidants during oocyte maturation increases cytoplasmic maturation and leads to higher rates of IVF and embryo development [205]. In my study, the antioxidant quercetin applied during IVM increased intracellular GSH levels and improved blastocyst development, which implies enhancement of cytoplasmic maturation. However, no beneficial effect of taxifolin treatment during IVM was found on first polar body extrusion rate, showing that it

could not affect nuclear maturation even though it effectively reduced ROS levels. Also, my observations that antioxidants, specifically quercetin, applied during oocyte maturation increases blastocyst formation, are in agreement with previous findings [95, 218-220].

In conclusion, treatment of porcine oocytes with the quercetin, a type of flavonoid, had a significant positive effect on embryonic development and reduced ROS generation by increasing intracellular GSH levels at low concentrations, but it was detrimental at high concentrations. It is not clear whether this concentration of quercetin is optimal in pigs. Therefore, further studies are needed to determine the optimal concentration of quercetin and to ascertain its beneficial effects on further development of pig embryos.

**PART V**

**GENERAL CONCLUSION**

In this study, for improving the maturation and embryo developmental efficiency of porcine oocytes, the effect of different oxygen tension as culture condition on IVM and IVC of porcine oocytes was investigated and the treatment effects of antioxidants such as melatonin, quercetin and taxifolin, on oocyte maturation and comparative effects of different oxygen concentration on oocytes culture condition were estimated.

On culture of the different oxygen concentration (5 and 20%), because blastocyst formation rate was estimated higher on culture condition of low oxygen concentration, low oxygen concentration indicated beneficial effect from oxygen toxicity on *in vitro* culture. According to analysis of differences in gene expression between cumulus-oocytes complexes cultured under 5 or 20% oxygen, each oxygen condition altered the expression of genes in different patterns. Specially, it showed that gene concerned antioxidant response was upregulated in the high oxygen condition. It can be concluded the low oxygen concentration may alter the expression of multiple genes related to oocyte competence and improves embryo development.

Supplements of maturation media with 10ng/ml melatonin improved the maturation rate and developmental competence. Oocyte cultured with melatonin generated lower ROS levels compared to others. Also we detected a local expression of melatonin receptor I on surface of the cumulus and granulosa cells through the presence of MT1 melatonin receptor transcript. These results showed

the exogenous melatonin works effectively on porcine oocytes maturation by working as a direct antioxidants or indirectly binding to receptors.

As plant-derived flavonoids, quercetin and taxifolin have an antioxidant property, I investigated the treatment effect of these flavonoids on embryo development. The result of quercetin and taxifolin treatment on maturation media showed that quercetin at the 1 $\mu$ g/ml has beneficial effect on developmental competence, but there is not significant effect on taxifolin treatment. Also, oocytes treated with quercetin had significantly lower levels of ROS and higher level of GSH. In this concentration, quercetin has not worked as phytoestrogens. Based on these findings, I concluded that exogenous quercetin reduces ROS levels during culture process and is beneficial for subsequent embryo development.

In conclusion, all data showed that because oxygen toxicity generated from culture process can cause detrimental effect on oocyte development, low oxygen tension during embryo culture have beneficial effect on embryo development and supplementation of antioxidant into the culture media may improve developmental competence of porcine oocyte *in vitro* by reducing ROS levels.

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## 요약 (국문초록)

바이옰의학 연구에 있어 돼지의 유용한 특성으로 인해 돼지 배아의 체외생산은 매우 중요한 단계로 여겨진다. 그러나 아직까지 돼지의 체외배아 생산율은 체내에 비해 낮은 편이다. 체외 배양시 발생하는 ROS 와 같은 산소독성은 낮은 생산율을 야기하는 다양한 원인중의 하나이다. 본 연구의 목적은 난자의 체외배양간 산소농도를 달리하여 산소독성이 야기될 수 있는 배양조건을 개선하고 더불어 배양배지에 항산화제를 처리함으로써 돼지 배아의 체외 생산율을 높이기 위함이다. 이러한 목적을 위해 본 연구는 체외 성숙 및 배양에 다른 산소농도 (5% 와 20%) 상황하에서 난자의 발달 효과 및 유전자 발현 현상을 검증하고, 돼지 난자에 멜라토닌과 몇몇 플라보노이드 (퀘세틴, 탁시폴린)을 각각 처리하여 체외발달에 대한 효과를 검증하였다.

우선, 다른 산소농도 (5% 와 20%)하에서 돼지난자를 성숙시키고 배양시켰을 때, 난자의 핵 성숙률에는 별다른 차이를 보이지 않았으나 20% 산소농도에서 성숙시킨 그룹을 5% 산소농도에서 체외 배양시 20% 산소농도에서 배양한 그룹보다 배반포 발달률이 유의적으로 향상되었다. 더불어 각 배양조건하에서 다양한 유전자의 mRNA 발현량 변화를 관찰하였을 때 낮은 산소하에서는 높은 글루코스 흡수작용과 혐기성 해당작용에 관련된 유전자가 높게 발현되었고 높은 산소하에서는 유사분열촉진 작용과 항산화작용에 관련된 유전자의 발현이 높았다.

따라서 난자를 20% 산소하에서 성숙시키고 낮은 산소하에서 배양하면 난자의 발달에 관련된 유전자 발현을 높여 난자의 발달을 유의적으로 증진시킬 수 있을 것이다.

배양환경에서 발생하는 ROS 독성을 방지하기 위해 난자의 체외성숙 배지에 몇몇 항산화제를 첨가하여 난자의 체외성숙 및 발달률을 검증하였다. 우선, 항산화제의 일종인 멜라토닌 호르몬을 성숙 배지에 첨가하였을 때, 10 ng/ml 의 처리 농도의 멜라토닌에서 난자의 성숙 및 배아 발달에 좋은 효과를 보였으며 ROS 발생 레벨도 낮추었다. 또한 멜라토닌의 발현 상태를 검증해본 결과 난자 주변의 난구 세포 및 과립막 세포에서 멜라토닌 수용체-1 유전자의 발현이 검증되었다. 결과적으로 외인성 멜라토닌은 돼지 난자의 체외성숙에 유용한 효과를 보였으며 이는 난구세포 표면 수용체와의 결합에 의한 간접적인 효과이거나 강력한 항산화제로서의 역할로 인한 직접적인 효과일 것으로 보인다.

또한 항산화제로서 플라보노이드의 일종인 퀘세틴을 성숙배지에 첨가하였을 경우 난자의 성숙에는 별다른 효과를 보이지 않았으나 지속 배양시 1 ug/ml 농도의 처리군에서 배반포 발달률이 유의적으로 향상되었다. 더불어 이 농도에서 난자는 낮은 ROS 발생레벨을 나타내었다. 그러나 고농도의 퀘세틴 처리는 오히려 난자 발달에 해를 끼치는 것으로 나타났다. 타키폴린 또한 플라보노이드로서 항산화 효과를 가지며 퀘세틴보다 독성이 낮은 것으로 알려져, 이후 두 인자를

성숙배지에 처리하여 그 효과를 비교하였다. 처리결과 50ug/ml 의 농도의 탁시폴린 처리군에서 난자에 유해한 효과를 보였다. 두 인자의 처리군을 비교한 결과 1 ug/ml 의 퀘세틴은 배반포 발달률 향상 효과를 보인 반면 1 ug/ml 의 탁시폴린은 유의적인 차이를 나타내지 않았다. 난자와 배아의 ROS 및 GSH 발생 레벨을 측정한 결과, 퀘세틴과 탁시폴린 처리그룹 모두 ROS 레벨을 낮추었으며 퀘세틴 처리그룹에서만 GSH 발생 레벨의 유의적인 차이를 나타내었다. 이 농도에서 퀘세틴과 탁시폴린 모두 식물성 에스트로젠으로서의 작용은 없었다. 결과적으로 퀘세틴과 같은 플라보노이드는 난자내의 ROS 발생레벨을 낮추어 체외발달에 효과적으로 작용한다.

이상의 결과를 종합하면, 체외배양간 산소농도의 감소는 돼지 난자의 체외배양 효율을 증대시킬 수 있었으며, 멜라토닌이나 퀘세틴과 같은 외인성 항산화제를 돼지 난자의 체외 배양 배지에 처리함으로써 배양기간 중에 발생하는 ROS 레벨을 낮추어 난자의 성숙 및 발달을 증진시킬 수 있었다.

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주요어 : 돼지 난자, 체외 성숙, 체외 배양, 항산화제, ROS

학번 : 2005-23754



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수의학박사학위논문

**Oxygen tension and Antioxidants  
on development of porcine *in vitro*  
produced embryos**

산소분압 및 항산화제에 의한  
돼지 체외배아 발육 향상에 관한 연구

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수의학과 수의산과·생물공학 전공

강 정택

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# **Oxygen tension and Antioxidants on development of porcine *in vitro* produced embryos**

**By JungTaek Kang**

**A THESIS SUBMITTED IN PARTIAL  
FULFILLMENT OF THE REQUIREMENT FOR  
THE DEGREE OF DOCTOR OF PHILOSOPHY**

**in**

**Theriogenology and Biotechnology  
Department of Veterinary Medicine, Graduate School  
Seoul National University**

**We accept this thesis as confirming to the required standard**

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**Seoul National University**

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## *Declaration*

*This thesis is submitted by the undersigned for the examination for the degree of Doctor of Philosophy to Seoul National University. This thesis has not been submitted for the purpose of obtaining any other degree or qualification from any other academic institution.*

*I hereby declare that the composition, work and experiments of this thesis are entirely my own.*

*JungTaekKang*



## **ABSTRACT**

# **Oxygen tension and Antioxidants on development of porcine *in vitro* produced embryos**

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Because of availability of pigs in biomedical research, *in vitro* production of porcine embryos is very crucial step. The oxygen toxicity resulted from ROS on *in vitro* environment could be one among numerous causes of low *in vitro* productivity. The purpose of this study is to investigate effects on embryo culture as change of culture condition resulted from different oxygen tension and to increase *in vitro* production rates of porcine embryos by supplementing some antioxidants to the media for defending of oxygen toxicity. So this study was conducted to investigate the effect of different oxygen concentration (5 and 20%) during *in vitro* maturation (IVM) and *in vitro* culture (IVC) and gene expression pattern, and to investigate effects of some antioxidants such as melatonin,

flavonoids (quercetin and taxifolin) on porcine oocyte maturation and embryo further development.

First, as a result of the effect of two oxygen concentrations during IVM and IVC on porcine embryo development, there were no significant differences in oocytes nuclear maturation rate. However, on further culture in 20% IVM, the 5% IVC group showed significantly increased blastocyst formation rate compared to the 20% IVC group. According to mRNA abundance data of multiple genes, each treatment altered the expression of genes in different patterns. As a result, in low oxygen, it occurred with a higher glucose uptake and an increase in anaerobic glycolysis in the cumulus cells, whereas in high oxygen, it happened to a higher activity of mitosis-promoting factor and antioxidant response in cumulus cells. Therefore, it can be concluded that high oxygen concentration during IVM and low oxygen during IVC may alter the expression of multiple genes related to oocyte competence and significantly improves embryo development.

Second, as one of means to supplement of antioxidants, melatonin was added to maturation media. Melatonin at the 10 ng/ml concentration during maturation showed the beneficial effect on the maturation rate and further developmental competence and lower levels of ROS. Also, as results of the local expression of the endogenous melatonin, melatonin receptor-1 gene expressed in cumulus and granulosa cells surrounding on oocytes. I concluded the exogenous melatonin has beneficial effects on nuclear and cytoplasmic maturation during porcine IVM. But

it is not clear whether the observed effects may be mediated by receptor binding or receptor independent, as a direct free radical scavenging.

The result of some flavonoids (quercetin and taxifolin) treatment as other antioxidants showed all quercetin and taxifolin treatment did not improve nuclear maturation of oocytes, but a significantly greater proportion of parthenogenetically activated oocytes developed into blastocysts when the IVM medium was supplemented with adequate quercetin (1 $\mu$ g/ml). As measurement result of levels of ROS and GSH in oocytes and embryos produced in maturation medium supplemented with quercetin or taxifolin, both treatment groups had significantly lower levels of ROS than controls, however GSH levels were different only in quercetin treated oocytes. I concluded that exogenous flavonoids such as quercetin reduce ROS levels in oocytes and may work effectively on embryonic development.

In conclusion, I suggested that low oxygen tension during culture effectively contribute to *in vitro* embryo development of porcine oocytes and that media supplemented with exogenous antioxidant such as melatonin and quercetin on the maturation process of porcine oocyte could promote maturation rate *in vitro* by reducing ROS level arisen during *in vitro* culture.

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Keywords : porcine oocyte, *in vitro* maturation, *in vitro* culture, antioxidant, oxygen tension, ROS

**Student number : 2005-23754**

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## LIST OF ABBREVIATIONS

<b>6-DMAP</b>	6-dimethylaminopurine
<b>BSA</b>	Bovine Serum Albumin
<b>COC</b>	Cumulus-oocyte complex
<b>DPBS</b>	Dulbecco's Phosphate Buffered Saline
<b>EGF</b>	Epidermal growth factor
<b>FBS</b>	Fetal bovine serum
<b>FSH</b>	Follicle stimulation hormone
<b>HEPES</b>	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
<b>ICSI</b>	IntraCytoplasmic Sperm Injection
<b>IVC</b>	<i>In vitro</i> culture
<b>IVM</b>	<i>In vitro</i> maturation
<b>LH</b>	Luteinizing Hormone
<b>PBS</b>	Phosphate Buffered Saline
<b>PCR</b>	Polymerase Chain Reaction
<b>PFF</b>	Porcine Follicular Fluid
<b>PVA</b>	Polyvinyl Alcohol
<b>PZM-3</b>	Porcine Zygote Medium-3
<b>ROS</b>	Reactive Oxygen Species
<b>RT</b>	Reverse Transcript
<b>SCNT</b>	Somatic Cell Nuclear Transfer
<b>TALP</b>	Tyrodé's albumin lactate pyruvate
<b>TCM</b>	Tissue Culture Medium



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# **PART I**

## **GENERAL INTRODUCTION**

# **1. LITERATURE REVIEW**

## **1.1 Pig as a scientific resource animal**

Recently, pigs have increasingly become utilized as biomedical research models. This increased use as an animal model is not only a result of regulatory pressure on other animal species such as rodents and primates, but also because pigs are recognized as a suitable animal model for human disease based upon their similarity in aspect of anatomy and physiology.

As advantages of pigs compared to other animal models, pigs are highly reproductive displaying early sexual maturity with 5~8 months, a short generation interval of 12 months, parturition of multiple offspring, an average of over 10 piglets per litter, and all season breeding [1]. Nowadays, standardization of the environment such as pig housing, feeding, and hygiene management, is well developed [2]. Also, reproductive technology and techniques of genetic modification have considerably advanced recently. Therefore, pigs are considered as pretty useful laboratory animal.

The human and pig have a large number of similarities in anatomy, physiology, metabolism, and pathology. Over the last a few decades, pigs have replaced other animal models as the general surgical model in the area for both training and research. Specially due to both anatomical and physiological similarity, the pig has been used successfully in studies of the cardiovascular system [3]. Pigs are used as

general cardiovascular surgical models including atherosclerosis. Also, because the physiology of digestion in omnivorous swine is remarkably like that in humans, pigs have become an increasingly important animal model for pharmaceutical applications for gastrointestinal system [4]. Furthermore, although porcine insulin differs from human insulin by only one amino acid, pig islet cells are functionally similar to humans, so pig islet may transplant to diabetes-patients [5]. Alike a rat, mouse, and rabbit, pig skin has been shown to be the most similar to human skin [6]. Pig skin is structurally similar to human epidermal thickness and dermal-epidermal thickness ratios, so they are especially useful in studying wound healing and burn lesions [7]. Like this in many human disease studies, pigs have been used for a long time.

Mainly, livestock pig breeds and miniature pigs are relevant models in many fields of medical research [8]. But there exist some differences between domestic farm breeds and miniature breeds. The differences are related to their growth rate and size at sexual maturity rather than actual anatomic differences in organs and structures. Intense breeding efforts have provided pig breeds differing substantially in important traits such as size, metabolic characteristics, and behavior. Standard farm pigs could be used in biomedical research protocols, but their use is typically limited to short-term studies because of their rapid growth rate, high feed intake, and large adult weight (280~450 kg). Specially bred-for-research minipigs are available from several sources and have proven to be valuable research animals due to the relatively slow growth rate and decreased adult weight (70 to 120 kg). Some minipig breeds that are currently available include the Yucatan, Hanford, Sinclair,

and Pitman-Moore. Taken together, it makes minipigs easier to use in studies more than farm pigs.

Moreover, if livestock pig breeds are employed for experimentation, the genetic background is mostly not defined. In contrast, minipig outbred stocks with full pedigree are delivered from commercial suppliers. In addition, inbred minipigs are available [9, 10]. Some pig breeds are used as non-rodent models for pharmacological and toxicological studies and are fully accepted by regulatory authorities worldwide.

The evolutionary gap between human and animal models such as rodents has always interfered with an applicability of information gained from many animal experiments to the human therapy. In this regard, the pig model offers many advantages [11]. In other words, the pig is a member of the artiodactyls (cloven-hoofed mammal) which are evolutionarily distinct from the primates and rodents [12], but there is extensive conserved homology between the pig genome and the human genome. The first draft of the porcine genome was generated by the “Sino-Danish Pig Genome Project” in 2005 using shotgun sequencing [13]. This initial evolutionary analysis based on 3.84 million shotgun sequences (0.66X coverage) from the pig genome and the available human and mouse genome data revealed that for each of the types of orthologous sequences investigated (e.g., exonic, intronic, intergenic, 5' UTR, 3' UTR, and miRNA), the pig is much closer to human than mouse [13]. This was confirmed by the comparative analysis of protein coding sequences using full-length cDNA alignments comprising more than 700 kb

from human, mouse, and pig where most gene trees favored a topology with rodents as outgroup to primates and artiodactyls [14]. To date, the first high coverage of the pig from a Duroc sow genome (*sus scrofa*) has been released through Ensembl ([http://www.ensembl.org/Sus\\_scrofa/Info/Index](http://www.ensembl.org/Sus_scrofa/Info/Index)) [15]. According to this Ensembl information, the genome of the pig comprises 18 autosomes, with X and Y sex chromosomes. The genome size is similar to that of human at around 2.7 Gb.

Finally, pigs are increasingly used as animal models for biomedical research due to the medical needs. The severe shortage of available donor organs (e.g., heart, kidney or liver) for organ transplantation has become a life threatening problem for patients with organ failure or organ damage, so making pig-to-human transplantation have come to the fore as an obvious solution for this problem [16]. But there are several obstacles to be overcome before this goal can be achieved. Preclinical studies which porcine heart or kidney was transplanted to primates showed that they created broadly complex rejection responses [17]. Also, pigs have emerged as potential sources of islet transplantation for clinical purpose. Wild-type porcine islets transplanted into the portal vein have successfully reversed diabetes in nonhuman primates [18]. But because of incomplete result of porcine islet transplant, there have been studies by using genetic engineered porcine model to overcome a few obstacles [19]. In addition, the pathogenesis, prevention and treatment of many human diseases (e.g., Alzheimer's disease, breast cancer, cardiovascular diseases) are still poorly understood while the incidences of those diseases are increasing yearly. The use of currently available rodent models for

studying human diseases is in some cases limited due to the many obvious differences between rodents and humans. Pigs are thus becoming an alternative animal model for studying human diseases [20].

Taken together, pigs as a scientific research model were developed to provide many investigators, veterinarians, technicians and others with the best possible medical source for biomedical purposes. As this resource expands gradually, the progressive development of human medical care will be approached in the near future.

## **1.2. *In vitro* maturation system of porcine oocytes**

The development of new techniques in pig reproduction such as transgenesis and cloning, creates a large demand for oocytes and embryos. Thus oocyte maturation is a critical component of IVP of embryos. *In vitro* maturation of oocytes is conducted above all things to gain the oocytes available to use other multiple procedures *in vitro* such as IVF, ICSI and SCNT. The goal of IVM is to get the *in vitro* oocytes progressed from the diplotene stage of prophase I (germinal vesicle or GV) to metaphase II (M II), along with cytoplasmic maturation that encompasses a broad set of intracellular events, all of which are essential for the fertilization and early development of the embryo [21]. Oocytes recovered from ovaries collected at the slaughter house were on immature state in both nuclear and cytoplasmic aspects. So it is important for immature oocytes to be matured to conduct further developmental process and usage. Since pig oocytes can be matured and this IVM oocytes can be fertilized *in vivo* [22], piglets were gained successfully from IVM-IVF oocytes [23]. But, although a great deal of progress has been made during last several decades, this current IVM systems still suffer from major several problems. First, it exist a low rate of development of IVM oocytes to the blastocyst stage and their low quality compared with *in vivo* produced embryos. Also in IVF process, a low rate of male pronucleus formation and a high rate of polyspermy in porcine oocytes [24].

It is well-known that the developmental competence of oocytes derived from small follicle (<3mm in diameter) to reach the M II stage after IVM is lower than

that of oocytes from medium follicle (3~6mm) [25]. According to recent research to examine the comparative competence of mature oocytes aspirated from small follicles and medium follicles of slaughterhouse-derived gilt ovaries, oocytes from small follicles are lower on the maturation rate compared to oocytes from medium follicles. But matured oocyte from two follicles had similar fertility ability *in vitro* and relative transcript abundance of concerned genes [26]. So, further studies are needed to determine the exact stage of ovary and the size of follicles on oocyte competence.

In oocyte morphology, pig oocytes form cumulus-oocyte complexes (COCs). Oocytes are selected using a criterion such as their morphology, including the numbers of cumulus cell layers surrounding the oocytes and uniform of the cytoplasm [27]. Normally, COCs with homogeneous ooplasm and a compact cumulus cell mass have been collected among many oocytes recovered from ovaries. COCs selected for collection process sometimes varies in quality and morphology according to subjective investigations. To prevent this asynchronous maturation due to subjective selection, recently the brilliant cresyl blue (BCB) test has been used successfully to select homologous oocytes for IVP [28]. However, according to recent research on chromosomal aberrations in oocytes selected by BCB test, it showed that BCB test rather disturb nuclear maturation of porcine oocytes [29].

Oocytes in culture are affected by specific physical conditions such as osmolarity, ionic composition, temperature, pH, CO<sub>2</sub>, and O<sub>2</sub> tension as well as maturation



media containing diverse supplements. In order to achieve *in vitro* matured porcine oocytes, media supplemented with serum or porcine follicular fluid (PFF), however, they contain many unknown factors and there is often considerable variability among sources or even among batches from the same source. Furthermore, there is a possibility that these fluids may contain hidden viruses [30]. Several reports have demonstrated that it is possible to replace serum or PFF with other defined compounds for maturation medium without reducing the efficiency on IVP embryo development [31, 32]. In the latter study, successful IVP in the defined system using a chemically defined medium was described in pig [33], and successful piglet production has been reported in the defined system [34].

Various basic culture medium types have been used for IVM of pig oocytes including North Carolina State University (NCSU) medium [35], modified tissue culture medium (TCM) 199 and modified Tyrode's medium containing lactate and pyruvate (mTALP) [36]. Many researches have attempted to overcome the low rate of IVM by providing conditions more similar to the *in vivo* maturation. The supplementation of various hormones in IVM medium has been performed and this has shown beneficial effects on oocytes maturation, such as follicle-stimulating hormone (FSH) [37], luteinizing hormone (LH) [38], pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotropin (hCG) [39], estradiol-17 $\beta$  [40], leptin [41] and relaxin [42]. Moreover, increasing studies have investigated the growth factors and many other factors that improve oocyte maturation, such as nerve growth factor (NGF) [43], brain-derived neurotrophic factor (BDNF) [44], growth differentiation factor 9 (GDF-9) [45], TGF- $\alpha$  [46],

epidermal growth factor (EGF) [47], EGF-like peptides [48], complement component 3 (C3) derivatives [49], L-carnitine [50] and sonic hedgehog [51].

Many researches suggest practical ways to solve the problems mentioned above, that is low rate of maturation and quality compared to *in vivo* counterpart.

### 1.3. *in vitro* embryo culture (IVC)

*In vitro* culture of embryos is an important procedure for improving the developmental competence of *in vitro* embryos produced by artificial process such as SCNT, IVF and ICSI. However, compared with *in vivo* production embryo, the overall efficiency of the pig embryo IVP technology is still extremely low and blastocysts obtained after IVF, SCNT and IVC is inferior in cell numbers and their ability to produce alive litters [52]. In addition, IVP embryos are characterized by a higher mean number of nuclei exhibiting DNA fragmentation per blastocyst compared to those produced *in vivo* [53]. Cytogenetic analysis of pig blastocysts produced *in vitro* showed that chromosomal aberrations occurred more higher in these blastocysts compared to embryos obtained *in vivo* [54, 55]. In general, the transfer of mammalian embryos produced *in vitro* results in considerable developmental abnormalities such as increased embryo mortality, extended gestation and considerably greater body weight of progeny [56, 57].

Although there are many causes for the developmental failure of IVP embryos, chromosomal abnormalities appear to be a major cause of this problem. According to study to determine the relationship between embryonic development speed at different stages and incidence of chromosome abnormalities in *in vitro* produced porcine embryo, the chromosomal abnormalities was significantly occurred higher in the blastocyst derived from 2-cell and >8-cell stage embryos than in the blastocyst derived from the other stage embryos. It indicated that selection of the best quality IVP embryos demands evaluation of embryo developmental

competence from early stages to parturition [55].

However, suboptimal culture conditions remain the major factor affecting the yield and quality of embryos obtained *in vitro*. Many culture media have been tested to optimize these conditions [58, 59]. Currently, the most commonly used media have a precise defined chemical composition and, in addition to salts and proteins, they contain energy sources such as glucose, calcium lactate, pyruvate and amino acids. Several media such as NCSU-37 Medium [60], modified synthetic oviduct fluid (mSOF) [61] and porcine zygote medium (PZM) [62] are available for the successful culture of embryos to the blastocyst stage.

Although a defined porcine IVP system has recently been developed by using PVA instead of PFF during IVM [33], but the most common component of culture media is the protein in the form of BSA or FBS. A beneficial effect of serum addition on the development of embryos has been reported in pigs [63]. Porcine embryos cultured with FBS to the blastocyst stage survived cryopreservation better than those that were not ( $P < 0.05$ , 42.9% vs. 28.6% respectively) [64]. And it has reported successful piglet production by IVF of oocytes matured *in vitro* using NCSU-37 supplemented with FBS [65]. Moreover, pig embryos were cultured in NCSU-23 medium supplemented with BSA significantly increased the proportion of morula and blastocysts production and decreased the average number of apoptotic nuclei and DNA fragmented nucleus index of blastocysts as compared to protein-free group (control) [66].

However, the difficulty to standardize culture conditions with the use of serum or serum albumin as well as infection risk have caused a growing interest in the development of medium with a strictly defined chemical composition. The use of these medium might provide more reproducible culture conditions and eliminate the presence of non-specific or pathogenic factors. One study attempted to replace BSA with PVA in adding to PZM media as macromolecular components [62]. In this study, total cell numbers in embryo cultured in PZM with PVA were greater than other media. The results of experiments on *in vitro* development of pig embryos in medium with animal protein substitute are encouraging and prompt further research. Also, addition of amino acids like glutamine and hypotaurine to culture medium promoted the development of embryos in pigs [67, 68].

Some study showed that exogenous supplement into culture condition is available to improve development of *in vitro* cultured embryos and make a higher blastocyst formation rate. For example, vitamin E, the predominant lipid-soluble antioxidant in animal cells, was considered as a major ROS scavenger which can block lipid peroxidation in cell membranes [69], therefore, it was predicted vitamin E could improve the development of porcine IVF embryos by suppressing cell membrane injuries caused by ROS [66]. Yuh et al.[70] have reported that the effect of vitamin E supplementation in NCSU-23 medium on porcine parthenogenetic embryos, it increased the average number of total cells at the blastocyst and decreased apoptotic cells at blastocyst as compared to control (without supplementation). In addition, it increases the proportion of porcine embryos reaching the blastocyst stage, reduces the average number of apoptotic nuclei and

DNA fragmentation nuclei [66] and the triglyceride content [71] in pig blastocyst after IVC. These results are agreement with previous reports the positive effects of vitamin E on the normal proportion and quality of cultured bovine blastocysts and on the accumulation of lipids in these blastocysts [72].

Recently, to facilitate nuclear reprogramming and thus improve cloning efficiency, several methods treating early nuclear transferred embryos with DNMT1 inhibitors like 5-aza-20-deoxycytidine (5-aza-dC) [73] and histone deacetylase inhibitors (HDACi) like TSA [74], scriptaid [75] and oxamflatin [76], have been tested to assist the somatic nucleus to mimic DNA methylation and chromatin remodeling. They found beneficial in improving cloning successful rate and correcting gene expression in pigs.

Due to many attempts to improve IVP of porcine embryos, now porcine embryos can now provide viable sources more efficiently with less cost and time compared with the *in vivo* counterparts.

#### 1.4. Reactive Oxygen Species

During a process of the energy generation in mitochondria, reactive oxygen species (ROS) are produced as product of the metabolism of oxygen in addition to energy. ROS are chemically reactive molecules containing oxygen. In normal situation, ROS have important roles in cell signaling and homeostasis [77]. However, during times of changeable environment like UV or heat exposure as well as conditions of internal and external oxidative stress, ROS levels can increase dramatically. This may result in significant damage to cell structure such DNA, RNA and proteins and contributes to the physiology of cellular senescence. Commonly, this is known as oxidative stress. Generally, oxidative stress results due to the loss of balance between ROS production and antioxidant defenses [78]. For example, oxidative damage resulted by  $\text{H}_2\text{O}_2$  can be ameliorated by catalase and superoxide dismutase by converting these compounds into oxygen and water, benign molecules. However, if this conversion is not totally efficient, residual peroxides will persist in the cell. So, excessive amounts of ROS can cause deleterious effects to DNA, RNA and protein in the cell [79].

ROS mainly include superoxide anion radical ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $^{\cdot}\text{OH}$ ), so on, concerned with process of reducing electrons gradually. These ROS are highly reactive to remove the electron from other molecules. In general, deleterious attacks from excess ROS may ultimately end in cell death and necrosis. These harmful attacks are mediated by the following more specialized mechanisms. First, opening of ion channels was started. And then lipid

peroxidation, oxidations of polyunsaturated fatty acids in lipids, occurred. Subsequently, oxidation of amino acid in proteins was worked. Finally, DNA oxidation was happened [80].

Current studies demonstrate that the accumulation of ROS can particularly decrease an organism's fitness in brain because oxidative damage is a contributor to senescence. In other words, the brain is particularly vulnerable to oxidative damage. Specially, due to the accumulation of oxidative damage, cognitive dysfunction is caused in rat experiment [81]. Accumulating oxidative damage can then affect the efficiency of mitochondria and further increase the rate of ROS production [82]. The accumulation of oxidative damage may be an etiology of human degenerative disease such as Alzheimer's disease. Many researchers accepted that oxidation of cellular proteins is potentially important for brain function [83].

Similarly, oxidative stress is caused in female reproductive system. This stress ratio can be altered by increased levels of ROS or a decrease in antioxidant defense mechanisms [80]. Excessive ROS production may hamper the body's natural antioxidant defense system of female, creating an environment unsuitable for normal physiological reactions. This, in turn, can lead to a number of reproductive disease including endometriosis, polycystic ovary syndrome, and unexplained infertility. It can also cause complications during pregnancy, such spontaneous abortion, recurrent pregnancy loss, preeclampsia, and intrauterine growth restriction. Moreover, in steroidogenic tissues such as the ovary, steroidogenic enzymes could be also sources of ROS [84].



Antioxidants are scavengers that detoxify excess ROS, which helps maintain the body's delicate oxidant/ antioxidant balance. There are two types of antioxidants : enzymatic and non-enzymatic. Enzymatic antioxidants possess a metallic center, which gives them the ability to take on different valences as they transfer electrons to balance molecules for the detoxification process. They neutralize excess ROS and prevent damage to cell structures. Endogenous antioxidants enzymes include SOD, catalase, glutathione peroxidase (GPx) and glutathione oxidase [85]. The non-enzymatic antioxidants consist of dietary supplements and synthetic antioxidants such as vitamin C, taurine, hypotaurine, vitamin E, Zn, selenium, beta-carotene and melatonin.

Also, ROS may originate from embryo metabolism and/or embryo surroundings. Embryo metabolism generates ROS via several enzymatic mechanisms. Generated ROS can induce development block and retardation [86]. Many antioxidants can alleviate oxidative stress during reproductive processes, and can enhance embryonic development *in vitro*. In pigs, as in other mammals, several antioxidants have been used as supplements in culture media to enhance embryonic development. These are as in the following; Ascorbic acid (vitamin C) [87], tocopherol (vitamin E) [88], selenium [89], insulin-transferrin-selenium (ITS) [90],  $\beta$ -carotene [91], retinoic acid (vitamin A) [92], lycopene [93], biochanin A [94] and anthocyanin [95]. Like this, many studies have continued its efforts that reduced ROS generated from metabolism by adding exogenous antioxidants during culture of embryo in pig.

## 2. OBJECTIVE

The purpose of this study is to investigate effects on porcine embryo as change of culture condition resulted from different oxygen tension and to increase *in vitro* production rates of porcine embryos by supplementing some antioxidants to the media for defending of oxygen toxicity. So this thesis is comprised of two part, one is to investigate the development competence of embryos during IVM and IVC on different oxygen tension, and the other is to investigate the effect of some antioxidants supplement into IVM media on the embryonic development. For the effect of different oxygen tension, porcine oocytes were matured in the two oxygen concentration (5% and 20%), and then cultured in the two oxygen concentration (5% and 20%), respectively (Part III). Each groups were checked *in vitro* developmental rate and multiple gene expression patterns. In next part, for the effects of antioxidant supplementation, porcine oocytes were matured in media with melatonin (part IV, chapter 1), quercetin (part IV, chapter 2) and taxifolin (part IV, chapter 3), respectively, and then cultured for 7 days after parthernogenetical activation. In this part, *in vitro* developmental rate, ROS and GSH level were checked. Lastly, final conclusion in this thesis was described in part V.

**PART II**

**GENERAL METHODOLOGY**

## **1. Chemicals**

Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

## **2. Oocyte Collection and *In vitro* Maturation**

Pig ovaries were collected from a local abattoir and transported to the laboratory in 0.9% (w/v) NaCl solution at 25-30°C. Follicular contents from antral follicles (3-6 mm in diameter) were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. The contents were pooled in a conical tube at 39°C and allowed to settle for a few minutes. The sediment was aspirated and diluted with Dulbecco's PBS (D-PBS; Invitrogen, USA) containing 100 U/mL penicillin G and 100 mg/mL streptomycin sulphate (pen-strep; Invitrogen, USA). Cumulus-oocyte complexes (COCs) with intact compact cumulus cell layers were selected and washed 3 times in TCM-Hepes before being transferred to a modified TCM-199 supplemented with 10 ng/ml EGF, 0.57 mM cystine, 0.91 mM sodium pyruvate, 5 µg/ml insulin, 1% (v/v) pen-strep, 0.5 µg/ ml follicle stimulating hormone, 0.5 µg/ ml luteinizing hormone and 10% porcine follicular fluid. For the first 22h only, the IVM medium also contained Gonadotropin. The COCs were cultured at 38°C with 5% CO<sub>2</sub> at maximum humidity. After 44 hr of maturation, oocytes were denuded of cumulus cells by pipetting with 0.1% hyaluronidase in D-PBS supplemented with 0.1% polyvinyl alcohol. Then denuded oocytes were treated according to each experimental design.

### **3. Assessment of meiotic maturation of matured oocytes**

The meiotic maturation was determined by evaluating for the presence of the polar body. After 44 hr of IVM, the denuded oocytes were fixed in methanol for 15 min, mounted on a slide and stained with Hoechst 33342 in D-PBS. The presence or absence of the polar body was determined under UV light.

### **4. Parthenogenetic activation of mature oocytes and *in vitro* culture**

At 44 hr of IVM, metaphase II oocytes were parthenogenetically activated. Briefly, denuded oocytes were equilibrated for 1 min in 0.26 M D-mannitol-based activation solution supplemented with 0.1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, and 0.5 mM HEPES. The oocytes were then transferred to a chamber between two electrodes spaced 3.2 mm apart and overlaid with activation solution. The oocytes were activated by electric stimulation with a single direct current pulse of 2.0 kV/cm for 60  $\mu$ sec using a BTX Electro-Cell Manipulator 2001 (BTX, USA). Parthenogenetically activated oocytes were cultured in 500  $\mu$ l porcine zygote medium-5 (Funakoshi, Japan) for 7 days at 39 °C in a humidified atmosphere with 5% CO<sub>2</sub> and 5% O<sub>2</sub>. The cleavage rate and blastocyst formation rate were checked at 48 and 168 hr of IVC, respectively.

In this study on embryonic development, I used parthenogenesis rather than *in vitro* fertilization or somatic cell nuclear transfer to assess oocyte competence for embryonic development. The parthenogenetic activation of oocytes can be used to evaluate the developmental competence of oocytes *in vitro* without confounding factors from spermatozoa [96] and a variety of other factors introduced during *in*

*vitro* procedures. Furthermore, parthenogenetic activation is relevant to cloning research, because artificial activation of an oocyte is an essential component of nuclear transfer protocols [97].

## **5. Assessment of embryo quality**

Blastocysts quality was assessed by Hoechst staining of the inner cell mass and trophectoderm cells according to standard procedures. Briefly, the blastocysts were washed in HEPES-buffered TALP medium and then incubated with TALP medium containing 25 µg/ml Hoechst stain for 15 min at 39 °C. The stained blastocysts were mounted onto glass slides under a cover slip and counted while examined with an inverted microscope (Nikon Corp., Japan) equipped to perform epifluorescence.

## **6. Measurement of intracellular GSH and ROS levels**

Oocytes were sampled after 44 hr of IVM and 2 d of IVC to determine intracellular GSH and ROS levels using CellTracker Blue CMF2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin) and the dichlorohydrofluorescein diacetate (DCHFDA) methods, respectively, previously described [95, 98] with slight modification. Briefly, 20 oocytes and embryos from each treatment group were incubated in the dark at 39°C for 30 min in TALP medium supplemented with 10 uM CellTracker and 10 uM DCHFDA. After incubation, oocytes were washed in HEPES-buffered TALP medium, placed on a glass slide and covered with a cover slip. The fluorescence emissions from the oocytes and embryos were recorded as TIFF files using a cooled CCD camera attached to a fluorescence

microscope (Axio Photo; Carl Zeiss Jena GmbH, Germany) with excitation filters (460nm for ROS and 370 nm for GSH). The recorded fluorescent images were analyzed using image J software 1.55 (National Institutes of Health, USA) by counting the number of pixels after color inversion [99].

## **PART III**

# **THE EFFECT OF DIFFERENT OXYGEN TENSION DURING EMBRYO CULTURE OF PORCINE OOCYTES *IN VITRO***



# **Chapter 1. Developmental competence of porcine oocytes after *in vitro* maturation and *in vitro* culture under different oxygen concentrations**

## **1. Introduction**

Pigs share many characteristics such as anatomy, physiology and body size with humans and are expected to become important animal models for therapeutic cloning and xenotransplantation [100, 101]. Because of this, IVM of oocytes and IVP of blastocysts in pigs are very important initial steps in the study of early embryonic development and the production of transgenic animals. Recently, the IVP of blastocysts in pigs was increased by technical improvements in several of the developmental stages [58]. However, the IVP rate is still inferior to *in vivo* embryo production, and the cloning efficiency after embryo transfer is still low [102]. To increase the rate of *in vitro* blastocyst formation and to improve cloning efficiency after embryo transfer, it is necessary to produce high-quality matured oocytes.

The oocyte and its surrounding cumulus cells in mammals are metabolically connected through gap junctions that serve as a unique means of entry into the ooplasm for several metabolites. Cumulus cells have a close connection with oocytes during the course of maturation. Gonadotropins, steroids and other factors

from the follicle cells also interact with oocytes to provide essential support for *in vivo* maturation of oocytes. It is generally accepted that cumulus cells support the maturation of oocytes to the metaphase II stage and are intimately involved in “cytoplasmic maturation” of oocytes, which is the capacity to undergo normal fertilization and subsequent embryonic development [103].

Generally, *in vitro* culture of porcine oocytes is maintained at higher concentrations of oxygen than the *in vivo* environment. Increased partial pressure of oxygen generates excessive amounts of cytotoxic ROS and may directly affect the viability of embryos [104]. Therefore, oxygen tension during culture would be an important factor affecting both oocyte maturation and embryo development. In cattle, several studies have shown that the efficiency of oocyte or embryo culture could be improved by decreasing oxygen concentration [105, 106], whereas others were contradictory [107, 108] or even showed that oxygen level had no significant effects on maturation and fertilization *in vitro* [109]. In mice, it was reported that a significant decrease in total cell number in blastocysts occurred as oxygen concentration increases during IVM [110]. In pigs, immature oocytes are commonly matured *in vitro* under a relatively high (20%) oxygen concentration although oxygen concentration is much lower than in the atmosphere both in the reproductive tract [111] and follicular fluid [112]. On the other hand, embryo culture is usually conducted *in vitro* under a relatively low (5%) oxygen concentration because fertilization occurs *in vivo* under low oxygen tension [113, 114]. In view of all these reports, it is clear that the effect of oxygen concentration during IVM or IVC is not yet fully understood.

The aim of this chapter was to observe porcine embryo development in terms of oocyte maturation and blastocyst formation derived from culture of these stages under different oxygen concentrations and to analyse the concomitant effects of oxygen on transcript abundance of genes related to metabolism, oxidative response, apoptosis and developmental competence in cumulus oocyte complexes and blastocysts.

## 2. Materials and methods

### 2.1 Oocyte collection and *in vitro* maturation

Detailed protocols were described in general methodology (Part II). After culture of 44 hr, the meiotic maturation was determined by evaluating for the presence of the polar body as described in general methodology. After each maturation period, oocytes were denuded from COCs by pipetting with 0.1% hyaluronidase in Dulbecco's PBS (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% polyvinyl alcohol (PVA-DPBS). Then denuded oocytes and separated cumulus cells were collected for total RNA extraction.

### 2.2 Parthenogenetic activation of matured oocytes and *in vitro* culture

*In vitro* culture of activated embryos was basically equal to that described as general methodology (Part II). Follows experimental design, a group of approximately 40 to 50 oocytes were cultured in 500  $\mu\text{l}$  PZM-3 supplemented with 4 mg/ml fatty acid-free BSA. Briefly, each maturation group was further subdivided into two groups for culture at 39 °C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% (C5) or 20% O<sub>2</sub> (C20), resulting in four groups (M5C5, M5C20, M20C5, M20C20). The cleavage rate and blastocyst formation rate were checked at 48 and 168 hr of IVC, respectively. Thus, the experimental design was a 2 x 2 factorial, with IVM and IVC x 5% and 20% oxygen. Blastocyst for RNA extraction and assessment of quality were randomly selected as equal number in each groups. The quality of blastocysts was assessed by Hoechst staining as general methodology.

### 2.3 Total RNA extraction, RT-PCR and Real Time PCR

Total RNA was isolated from fresh or previously frozen (-80°C) cumulus cells, oocytes matured either at atmospheric or low oxygen concentration (M20 or M5, respectively) and blastocysts obtained from oocytes matured at atmospheric oxygen concentration and cultured at atmospheric or low oxygen concentration (C20 or C5, respectively) using the easy-spin<sup>TM</sup> (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Korea) according to the manufacturer's instructions, and quantified by a spectrophotometer and immediately stored at -80°C until used for RT-PCR and qRT-PCR. cDNA (complementary DNA) was produced from 1µg of total RNA extracted from cumulus cells, oocytes and blastocyst samples, using a SuperScript<sup>TM</sup> III First-Strand cDNA Synthesis Kit (Invitrogen Life Technologies) primed with oligonucleotide-dT (18mer) and followed by RNase H digestion of RNA, in a total volume of 20 µl as per the manufacturer's instructions. Real Time PCR was done according to the TAKARA BIO INC. with little modification. In brief, all the primers were standardized by standard curve. The PCR plate (MicroAmp optical 96-well reaction plate, Singapore) was made by adding 2 µl cDNA, 1µl forward primer, 1 µl reverse primer, 10 µl SYBR Premix Ex Tag (Takara Bio Inc), 0.4 µl ROX Reference Dye (Takara Bio Inc.) and 5.6 µl of Nuclease-free water (Ambion Inc., Applied Biosystem). For each sample, four replications were made in a plate. The wells were capped by using MicroAmp optical 8-cap Strip, USA. The plate was then vortexed (Vortex-2, Genie, Scientific Industries Inc, Bohemia, N.Y. 11716, USA) and centrifuged briefly in a plate spinner (Plate Spin, Kubota). Real time PCR was done by using a 7300 Real Time PCR System (Applied Biosystems, Singapore)

according to the company instructions. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1 containing the specific primer to amplify glucose transport 1 (GLUT1), lactate dehydrogenase A (LDHA), glucose-6-phosphate dehydrogenase (G6PD), Mn-superoxide dismutase (MnSOD), glutathione peroxidase (GPX1), insulin-like growth factor receptor 2 (IGFR2), Bcl-2-associated X protein (BAX), B-cell lymphoma 2 (Bcl2), prostaglandin G/H synthase-2 (PTGS2, also known as COX2), the M-phase promoting factor subunit Cyclin B1 (CCNB1), Gremlin (GREM1), DNA-methyltransferase 3 beta (DNMT3B), aldo-keto reductase family 1 member B1 (AKR1B1), POU class 5 homeobox 1 (POU5F1) and caudal type homeobox 2 (CDX2).

## 2.4 Statistical analysis

Each experiment was repeated at least 4 times. All statistical analyses were performed using Prism4 software (GraphPad, U.S.A.). *t-test* was used for oocyte maturation and embryo development and Two-way analysis of variance (ANOVA) was used to determine significant differences in data levels followed by a Bonferroni post-tests to determine statistical differences of development rate and mRNA abundance among groups. Significant differences among the treatments were determined when  $P < 0.05$ . Data are expressed as means  $\pm$  S.E.M..

**Table 1.** Details of primers used for quantitative reverse transcription-polymerase chain reaction

Gene	Primer sequences (5' → 3')	Length of PCR product (bp)	Gene bank accession no. or References
<b>GLUT1</b>	F: 5'- GCTTCCAGTATGTGGAGCAACT R: 5'- AAGCAATCTCATCGAAGGTCC	132	X17058.1
<b>LDHA</b>	F: 5'- ATCTTGACCTATGTGGCTTGGA R: 5'- TCTTCAGGGAGACACCAGCAA	214	NM_001172363.1
<b>CCNB1</b>	F:5'- TTGACTGGCTAGTGCAGGTTC R:5'- CTGGAGGGTACATTTCTTCATA	368	NM_001170768.1
<b>Bcl2</b>	F:5'- TGGTGGTTGACTTTCTCTCC R:5'- ATTGATGGCACTAGGGGTTT	139	AF216205
<b>G6PD</b>	F: 5'- CCTCCTGCAGATGCTGTGTCT R: 5'- CGCCTGCACCTCTGAGATG	112	L.Jiang et al. [115]
<b>GPX1</b>	F: 5'- GATGCCACTGCCCTCATGA R: 5'- TCGAAGTTCCATGCGATGTC	80	AF532927
<b>IGFR2</b>	F: 5'- CGCTCTCTGCCTCTAGCAGT R: 5'- CCTACACCCCAAGTCTGGAA	225	AF342812
<b>MnSOD</b>	F: 5'- GCTTACAGATTGCTGCTTGT R: 5'- AAGGTAATAAGCATGCTCCC	101	S67818.1

<b>GREM1</b>	F: 5'- AACAGCCGTACCATCATCAAC R: 5'- TTCAGGACAGTTGAGAGTGACC	156	NM_001082450.1
<b>BAX</b>	F: 5'- GCCGAAATGTTTGCTGACGG R: 5'- CGAAGGAAGTCCAGCGTCCA	152	AJ606301
<b>PTGS2</b>	F: 5'- CTGCCGTGTCGCTCTGCACTG R: 5'- TCATAACTCCATATGGCTTGAAC	287	AY028583
<b>DNMT3B</b>	F: 5'- AGTGTGTGAGGAGTCCATTGCTGT R: 5'- GCTTCCGCCAATCACCAAGTCAAA	133	NM_001162404.1
<b>AKR1B1</b>	F: 5'- AAGGAGCACAGTTCCAAGCAGTCA R: 5'- CCCGAAGAGCACTACCTGTAGATT	166	CO994619
<b>POU5F1</b>	F: 5'- TTTGGGAAGGTGTTTCAGCCAAACG R: 5'- TCGGTTCTCGATACTTGTCCGCTT	198	NM_001113060
<b>CDX2</b>	F: 5'- TGTGCGAGTGGATGCGGAAG R: 5'- CCGAATGGTGATGTAGCGACTG	149	gi   262070767
<b><math>\beta</math>-actin</b>	F: 5'- GTGGACATCAGGAAGGACCTCTA R: 5'- ATGATCTTGATCTTCATGGTGCT	137	U07786

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### **3. Results**

#### **3.1 Effects of oxygen concentration on oocyte nuclear maturation**

A total of about 300 oocytes were used in eight replicates to evaluate the effects of 5% vs. 20% oxygen on nuclear maturation during IVM. The first polar body extrusion rate was not significantly difference between these two groups (Table 2).

**Table 2.** Effects of different oxygen concentrations during IVM on the frequency of first polar body extrusion

Concentration %	Oocytes, n	Oocytes with first polar body extrusion, n	First polar body extrusion rate (% $\pm$ S.E.M.)
<b>5</b>	310	258	83.22 $\pm$ 1.6
<b>20</b>	311	266	85.53 $\pm$ 0.8

First polar bodies were counted by Hoechst staining after 44 hr maturation *in vitro*.

No differences between groups were observed.

### 3.2 Effects of oxygen concentration on subsequent development of porcine oocytes *in vitro*

The number of blastocysts was significantly increased in the 5% IVC group vs. the 20% IVC group on further culture after maturation at atmospheric oxygen concentration. Moreover, the group matured under 20% oxygen and cultured under 5% oxygen (M20C5 group) displayed higher development than the other groups ( $P < 0.05$ ). No differences were observed among the M5C5, M5C20 and M20C20 groups. The oxygen concentration had no effect on the first cleavage frequency or the cell number per blastocyst (Table 3). Also, blastocyst morphology or degree of expansion was not different among the groups.

**Table 3.** Overall cleavage rate and blastocyst yield following maturation (M group) and further development in culture (C group) under atmospheric (20%) or low (5%) oxygen

Treatment	Embryos examined, n	Cleavage, n (%) <sup>*</sup> ≥2 cell	Blastocysts, n (% ± S.E.M.)	No. of cells per blastocyst (means ± S.E.M.)
<b>M5C5</b>	118	70 (59.32)	17 (14.41) <sup>a</sup>	34.33 ± 3.7
<b>M5C20</b>	117	72 (61.54)	12 (10.26) <sup>a</sup>	32.40 ± 1.6
<b>M20C5</b>	126	88 (69.84)	29 (23.02) <sup>b</sup>	31.83 ± 2.3
<b>M20C20</b>	123	85 (69.11)	22 (17.89) <sup>ab</sup>	33.20 ± 4.6

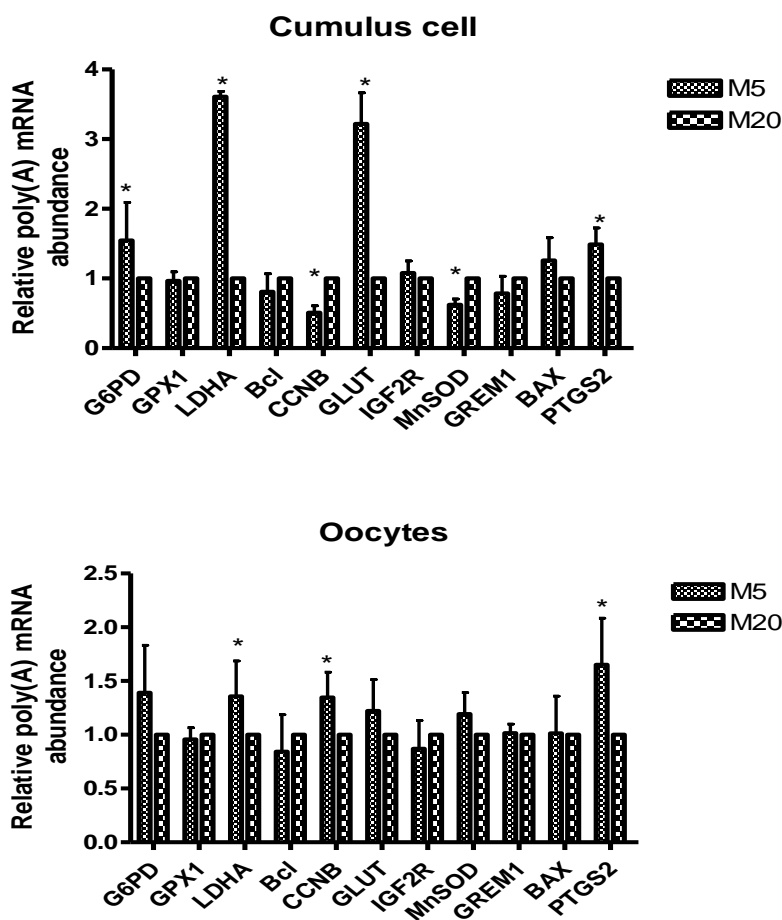
Percentages are based on the number of embryos examined.

<sup>a, b</sup> : Within the same column, values with different superscripts were different (P<0.05).

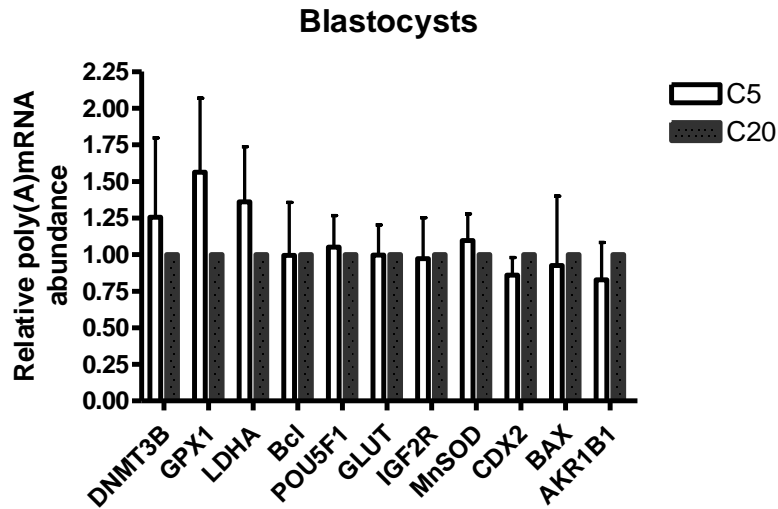
### 3.3 COC and blastocyst relative poly(A) mRNA abundance

The relative poly(A) mRNA abundance of eleven genes was analyzed in cumulus cells and oocytes, as shown in Figure 1. These genes were selected to analyze whether oxygen level affected biological functions essential to produce developmentally competent oocytes, such as glucose transport and metabolism, antioxidant response and apoptosis. Three of the genes analyzed are related to metabolism (GLUT1, LDHA and G6PD). GLUT1, LDHA and G6PD was up-regulated in cumulus cells matured in 5% oxygen compared with those matured under 20% oxygen ( $p<0.05$ ). Two of the genes analyzed are related to antioxidant response (MNSOD and GPX1). MnSOD was up-regulated in cumulus cells matured in 20% oxygen ( $p<0.05$ ). The growth factor IGFR2, proapoptotic gene BAX and anti-apoptotic gene BCL2 did not show significant differences among groups. Among genes selected for oocyte competence, the CCNB1 poly(A) mRNA content was significantly higher in cumulus cells matured under 20% oxygen, but the reverse was found in oocytes ( $p<0.05$ ). PTGS2 was up-regulated in cumulus cells and oocytes matured in 5% oxygen ( $p<0.05$ ). GREM poly (A) mRNA content showed no significant differences among groups.

In blastocysts resulted from parthenogenetically activated oocytes, I analyzed their quality in terms of transcription of additional genes (methylation : DNMT3B, blastocyst quality : AKR1B1, POU5F1 and CDX2) as well as the previously mentioned genes. No differences in mRNA abundance of these candidate genes were found among blastocysts derived from oocytes cultured under different oxygen concentrations (Figure 2).



**Figure 1.** Relative poly(A) mRNA abundance of 11 candidate genes related to metabolism (GLUT1, LDHA and G6PD), antioxidant response (MnSOD and GPX1), growth factors and apoptosis (IGF2R, Bcl 2 and BAX) and oocyte competence (CCNB1, PTGS2 and GREM1), in cumulus-oocyte complexes matured *in vitro* under 5% or 20% oxygen atmosphere. upper: cumulus cells, lower: oocytes. An asterisk (\*) indicates differences between groups based on one-way analysis of variance ( $P < 0.05$ ).



**Figure 2.** Relative poly(A) mRNA abundance of 11 candidate genes related with metabolism (GLUT1 and LDHA), antioxidant response (MnSOD and GPX1), growth factors and apoptosis (IGF2R, Bcl2 and BAX), methylation (DNMT3B) and blastocyst quality (AKR1B1, POU5F1 and CDX2), in blastocysts produced from embryos cultured under 5% or 20% oxygen atmosphere. No differences were observed.

## 4. Discussion

The purpose of this study was to determine the relationship between different oxygen concentrations and maturation rate or developmental competence of porcine oocytes. Some studies of porcine *in vitro* maturation systems reported that low oxygen concentration during IVM or IVC has a beneficial effect [116, 117], but others indicated that is more effective for IVM or IVC [118] or even that oxygen level has no significant effects on IVM or IVC [109]. As a result, the optimal oxygen concentration is a controversial issue in porcine embryo production systems. In this chapter, I evaluated quantitative changes of some genes in embryos matured under different oxygen concentrations. Oxygen level during IVM had no significant effect on oocyte maturation. However, atmospheric oxygen concentration during IVM followed by IVC at 5% oxygen significantly improved embryo development. These results suggest that the use of an appropriate oxygen tension during IVM and IVC may be particularly useful in techniques that demand highly competent oocytes, such as ICSI, SCNT and oocyte vitrification.

To evaluate the effect of oxygen concentration, the relative mRNA abundance of some genes related to embryo quality was analyzed, comprising three metabolism-related genes (GLUT1, G6PD and LDHA), two genes related to antioxidant response (MnSOD and GPX1), two genes related to apoptosis (Bcl2 and Bax), one related to growth factor (IGF2R), and three genes related to oocyte competence (CCNB1, PTGS2 and GREM1).



The glucose transporter GLUT1, the enzyme involved in anaerobic glycolysis, LDHA, and the enzyme that catalyses the first and irreversible step of the pentose phosphate pathway, G6PD, are important for metabolism. It was found that the poly(A) mRNA abundance of GLUT1, LDHA and G6PD were higher in the cumulus cells of COCs matured under low oxygen, implying a higher glucose uptake and an increase in anaerobic glycolysis. Most of the ATP for oocyte maturation is provided by glycolysis or imported from granulosa cells. Energy substrates required for oocyte maturation may differ under low and high oxygen. Both glucose and glutamine, alone or in combination, can support preimplantation development of pig embryos [119]. Glucose is metabolized equally via aerobic and anaerobic pathways, although glycolysis becomes the dominant pathway as development progresses. From this information, it is evident that pig embryos have changing metabolic needs as they develop and undergo the accompanying environmental changes such as oxygen tension.

To test possible differences in ROS production, poly(A) mRNA abundance of two genes related to oxidative response in both cumulus cells and oocytes (MnSOD and GPX1) was analyzed. The results suggest differences between treatments in antioxidant response according to poly(A) mRNA abundance reflected in different oxygen levels, specifically being higher under 20% oxygen. However, no differences were found in relative abundance of the proapoptotic gene BAX poly(A) mRNA or the anti-apoptotic gene Bcl2 poly(A) mRNA in cumulus cells and oocytes, suggesting no change related to apoptosis under different oxygen concentrations. ROS can induce apoptotic cell death in oocytes cultured without

cumulus cells [104]. Cumulus cells may effectively protect oocytes against apoptosis caused by ROS generated by high oxygen concentration.

Finally, the relative poly(A) mRNA abundance of three genes related to oocyte competence (CCNB1, PTGS2 and GREM1) was analyzed. A higher poly(A) mRNA abundance of CCNB1 was observed in cumulus cells matured under high oxygen concentration and in the oocytes under low oxygen, which may suggest a higher activity of mitosis-promoting factor (MPF). CCNB1 is the principal molecule for regulation of mammalian oocyte maturation and synthesis of CCNB1 is necessary for GVBD induction in a normal time course. That is, oocyte is not required for the activation of MPF during the first meiosis, but that it is required for the second meiosis because of its promotion of CCNB1 accumulation [120, 121]. PTGS2 mRNA abundance in human cumulus cells was higher from oocytes that developed into higher quality embryos compared with lower quality embryos [122]. In a study using PTGS2 knockout mice, full cumulus expansion was not observed in ovulated COCs and *in vivo* fertilization was completely suppressed [123]. In porcine, the expression of PTGS2 mRNA were up-regulated by FSH and LH during maturation period [124] and it has also been correlated with oocyte competence improvement. In the present study, a higher expression level of PTGS2 mRNA was found in cumulus cells and oocytes from COC matured under low oxygen concentration.

Expression patterns of AKR1B1, POU5F1, CDX2 and DNMT3B relevant to early embryonic development provide information to assess the quality of

blastocysts derived from IVM/IVC [125, 126]. Not only was total cell number without significant differences in blastocysts, but also no differences were observed in mRNA abundance of these genes or of other previously mentioned genes associated with metabolism, apoptosis and antioxidant response. These results show that although blastocyst production rate is significantly different, different oxygen concentrations during IVC had no effect on the quality of blastocysts.

During follicular development, oocyte quality is affected by communication between the oocyte and surrounding cumulus cells. Removal of cumulus cells before IVM decreases the quality of oocytes in pigs [127]. Therefore, cumulus cells are considered to have an important role in oocyte maturation by regulating meiotic progression and by supporting cytoplasmic maturation. However, the mechanisms by which cumulus cells improve oocyte maturation are poorly understood. In this study, poly(A) mRNA abundance of multiple genes in cumulus cells varied depending on the oxygen concentration during IVM. This means that cumulus cells play their important roles during IVM selectively under different oxygen concentrations. Under low oxygen, cumulus cells increase glucose metabolism via anaerobic glycolysis, but under high oxygen the cumulus cells increase endogenous antioxidants as protection against ROS production. Because of these beneficial effects of cumulus cells, oocytes probably could be matured *in vitro* with no significant difference in maturation rate regardless of oxygen concentration. But, because embryos are cultured without cumulus cells during IVC, they lack this protection *in vitro* and thus may be susceptible to harmful effects of high oxygen.

In conclusion, the use of low oxygen concentration during IVC significantly improves embryo development in terms of numbers, but not blastocyst quality in terms of mRNA abundance of associated candidate genes.

## **PART IV**

# **THE EFFECT OF ANTIOXIDANTS ON MATURATION AND CULTURE OF PORCINE OOCYTES *IN VITRO***

# **Chapter 1. Effects of Melatonin on *in vitro* maturation of porcine oocyte and expression of Melatonin receptor RNA in cumulus and granulosa cells**

## **1. Introduction**

*In vitro* matured porcine oocytes have proven to be useful sources for assisted reproductive technologies such as IVF, ICSI, and SCNT. Due to their physiological similarity to human, the pig is a good model species for human disease. Moreover, transgenic cloned pigs can potentially be used for xenotransplantation. However, compared to *in vivo* derived embryos, the *in vitro* developmental porcine embryo is still low [128]. Therefore, the application of cloning technique will require an increase in efficiency through refinements *in vitro* oocyte maturation and embryo culture systems.

*In vivo*, oocytes and embryos produce endogenous ROS by various pathways [129]. *In vitro* handling and culture expose oocytes and embryos to oxidative stress resulting from events such as exposure to light, elevated oxygen concentrations, and disturbed concentrations of metabolites and substrates [130]. The ROS such as hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^-$ ) anions, or hydroxyl radical ( $OH^\cdot$ ), can damage cell membranes [131] and DNA [132] and may play a role in apoptosis

[98]. Therefore, it is important to protect oocytes against oxidative stress during *in vitro* maturation. One approach is to supplement the medium with antioxidant compounds.

Melatonin (N-aceyl-5-methoxytryptamine), an indole derivative secreted rhythmically from the pineal gland, plays a major role in regulating the circadian clock in mammals [133]. Also, this hormone has major effects on the reproductive system in mammals [134, 135]. More recent studies have demonstrated that, besides its multiple actions on different physiological processes, melatonin is an indirect antioxidant and a powerful direct scavenger of free radicals [136-139].

In contrast to the majority of other known radical scavengers, melatonin is multifunctional and universal [140-142]. It is soluble both in water and in lipids and hence acts as a hydrophilic and hydrophobic antioxidant. It has been shown that melatonin and its metabolites directly scavenge hydroxyl radical, organic oxyl radical, peroxy radical, peroxyxynitrite anion, nitric oxide and singlet oxygen [143-146]. Also, melatonin may repair some molecules that have been oxidized [147].

Melatonin has been successfully tested for promoting mouse embryo development *in vitro* [148]. It has been also reported as having no detrimental effects on mouse or rat embryo development during toxicity tests [149-151] performed either *in vitro* or *in vivo* [151]. Recently, an increased ratio of vitrified sheep blastocysts developing with melatonin during 24 and 48 hr *in vitro* post warming culture was reported [152].

Taken together, these data suggest that melatonin might have beneficial effects as an antioxidant agent during porcine oocyte maturation and/or embryo development. Besides the need to verify its previously described effects, it is not clear what stages of porcine pre-implantation development are influenced by melatonin. The present study examined the effects of melatonin on porcine oocyte maturation and pre-implantation development of embryos *in vitro*. The specific objectives were to evaluate 1) effects of melatonin supplementation during IVM on the frequency of extrusion of polar bodies and parthenogenetic development of embryos, 2) determine effects of melatonin on the ROS levels of *in vitro* matured cumulus oocyte complexes, 3) investigate the local expression of melatonin receptor I in cumulus cells, granulosa cells and oocytes.



## **2. Materials and methods**

### **2.1 Experimental design**

In order to determine effective concentrations for improving IVM of the oocyte (Experiment 1), melatonin was supplemented in each IVM medium at four concentrations (0, 10, 50 or 100 ng/ml) during the entire maturation period of 44 hr (the first half, eCG- and hCG-containing TCM-199 medium for 22 hr; and the latter half, eCG- and hCG-free TCM-199 medium for 22 hr). In experiment 2, we evaluated the effects supplementing the IVM medium with melatonin at the four concentrations on the parthenogenetic *in vitro* development of embryos. In experiment 3, I assessed the effects of similar melatonin concentrations in IVM medium on the ROS levels. Experiment 1 was designed to determine the presence of melatonin receptor on oocytes and granulosa and cumulus cells.

### **2.2 Oocyte Collection and *in vitro* Maturation**

Detailed protocols were described in general methodology (Part II). But, TCM-199 supplemented with 0.1 % PVA was used instead of PFF as maturation media in this chapter. The meiotic maturation was determined by evaluating for the presence of the polar body as described in general methodology.

### **2.3 Activation of matured oocytes**

For parthenogenetic activation, electrical activation protocol described in general methodology (Part II) was used.

#### 2.4 *In vitro* culture of activated parthenogenetic embryos

*In vitro* culture of activated embryos was basically equal to that described as general methodology (Part II). Follows experimental design, a group of approximately 40 to 50 oocytes were cultured in 500  $\mu\text{l}$  medium containing PZM-3 supplemented with 4 mg/ml fatty acid-free BSA. The quality of blastocysts was assessed by Hoechst staining as general methodology.

#### 2.5 Isolation of porcine granulosa and cumulus cells, RNA isolation and RT-PCR

Porcine granulosa cells were isolated as previously described [153, 154]. Follicular aspirates from ovary were centrifuged at 250 g for 10 min at 4 °C. The supernatant was then aspirated, and cell pellets from ovary were pooled and resuspended in PBS, overlaid onto a 60% (v/v) Percoll solution and centrifuged at 1000 g for 20 min at 4 °C. Granulosa cells precipitating at the Percoll-PBS interface were aspirated, resuspended in PBS and centrifuged at 250 g at 4 °C. This step was repeated three times. After the final wash, the supernatant was removed and the cell pellet was resuspended in PBS. Cell viability was determined by the trypan blue dye exclusion method.

Cumulus cells were removed from matured oocytes by pipetting in 0.1% hyaluronidase. Denuded oocytes and their respective cumulus cells were washed once in PBS, and total RNA was isolated from fresh or previously frozen (-80°C) granulosa cell, cumulus cells or fresh granulosa cells, cumulus cells, and oocytes using the RNeasy total RNA kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions, and quantified by a GeneQuant II spectrophotometer (Pharmacia, Uppsala, Sweden). The yield of total RNA was about 2~3  $\mu\text{g}$  RNA per

million granulosa cells, cumulus cells and oocytes. cDNA was produced from 5 µg of total RNA per porcine granulosa cells, cumulus cells and oocyte samples, using a SuperScript II reverse transcriptase kit (Gibco BRL, Grand Island, NY, USA) primed with oligonucleotide-dT (18 mer) and followed by RNase H digestion of RNA, in a total volume of 20 µl as per manufacturer's instructions. PCR was performed as described previously [155], using the following oligonucleotide primer. Porcine MT I primer sequence is positive strand 5'-tattgctacatctgacacagtc-3' and negative strand 5'-gccacaaacagccactctggga-3'. The PCR conditions were changed by reducing MgCl<sub>2</sub> concentration to 1.5 mM and the cycling conditions were 93°C, 3 min, followed by 35 cycles of 94°C, 30 sec; 64°C, 1 min; 72°C, 45 sec; plus a 5-min extension at 72°C. The product was a 461 bp fragment.

## 2.6 Measurement of ROS contents

Oocytes were sampled after IVM to determine intracellular ROS levels as previously described in general methodology of Part II.

## 2.7 Statistical analysis

All statistical analyses were performed using Prism4 software (GraphPad, U.S.A.). One-way ANOVA was used to determine significant differences in data levels. Tukey test was followed to determine statistical differences between groups. The significant differences among the treatments were determined when  $P < 0.05$ . Data are expressed as means  $\pm$  S.E.M..

### **3. Results**

#### **3.1 Effect of melatonin on porcine oocyte nuclear maturation**

A total of about 889 oocytes were used in five replicates to evaluate the effects of melatonin on nuclear maturation during IVM. The polar body extrusion rate was significantly higher ( $P < 0.05$ ) in the group supplemented with 10 ng/ml ( $84.6\% \pm 4.10$ ) when compared with the control group ( $75.6\% \pm 6.6$ ) and groups (Table 4).

**Table 4.** Effects of melatonin supplementation during IVM on the frequency of polar body extrusion

Concentrations (ng/ml)	Oocytes, n	Oocytes with polar body extrusion, n	Polar body extrusion rate (% $\pm$ S.E.M.)
0 (Control)	221	167	75.57 $\pm$ 2.94 <sup>a</sup>
10	227	192	84.58 $\pm$ 1.83 <sup>bc</sup>
50	220	178	80.91 $\pm$ 0.96 <sup>ab</sup>
100	221	169	76.47 $\pm$ 1.81 <sup>a</sup>

Polar bodies were counted by Hoechst staining after 44 hr maturation *in vitro*.

<sup>a,b,c</sup> Different letters indicate statistically significant differences ( $P < 0.05$ ).

### 3.2 Effects of melatonin on further development of porcine oocytes *in vitro*

A total of 672 oocytes that underwent IVM in media supplemented with four concentrations of melatonin were parthenogenetically activated in four replicates. Melatonin supplementation had no effect on the first cleavage frequency and the cell number per blastocyst (Table 5). However, significantly greater ( $P<0.05$ ) frequency of blastocysts developed in oocytes when IVM medium was supplemented with 50ng/ml melatonin. Supplementation of IVM medium with melatonin improved, at least numerically, the frequency of blastocyst development (Table 5).

**Table 5.** Effects of melatonin on further development of porcine oocytes *in vitro*

Concentrations (ng/ml)	Oocytes examined, n	Cleavage, n (%) <sup>*</sup> ≥2 cell	Blastocyst, n (% ± S.E.M.)	No. of cells per blastocyst (means ± S.E.M.)
0 (Control)	163	122 (74.85)	22 (13.28±2.101) <sup>a</sup>	60.50 ± 19.96
10	171	131 (76.61)	32 (18.50±2.377) <sup>ab</sup>	47.47 ± 13.63
50	175	134 (76.57)	38 (21.38±1.869) <sup>b</sup>	53.85 ± 16.84
100	163	124 (76.07)	34 (21.07±1.546) <sup>ab</sup>	55.87 ± 17.37

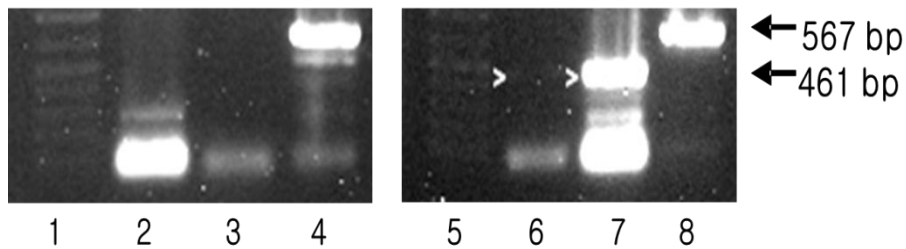
Percentages are based on the number of oocytes examined.

<sup>a, b</sup> : Within the same column, values with different superscripts were significantly different (P<0.05).

### 3.3 Expression of MT1 melatonin receptor mRNA expression in cumulus cells, granulosa cells and oocytes

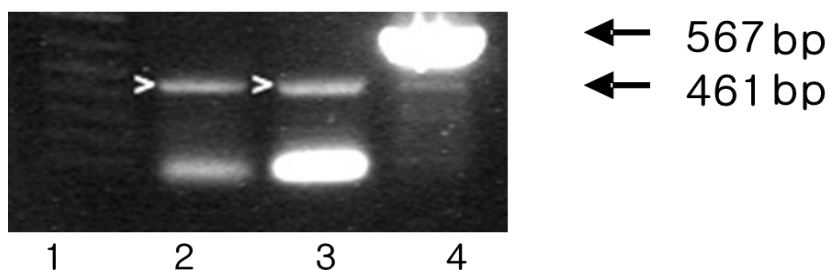
MT1 melatonin receptor cDNAs were amplified from mRNA isolated from porcine cumulus cells (Fig 3, right) and granulosa cells (Fig 4) by RT-PCR with two rounds of amplification. PCR products obtained after the second round of amplification using nested primers were of the expected size (461 bp) for MT1 melatonin receptor. MT1 mRNA expression was not detected in porcine oocytes. (Fig. 3, left).





**Figure 3.** RT-PCR analysis of MT1 melatonin receptor mRNA expression in the porcine cumulus cells and oocytes

DNA gel electrophoresis of first (lane 2 and 6) or second (lane 3 and 7) amplification products generated from porcine cumulus cells or oocytes. Lanes 1 and 5, DNA molecular weight marker (DNA-*Hae*IV). Using MT1 nested primers, a band of 461 bp corresponding to the expected size for the amplified product of the MT1 melatonin receptor was obtained in cumulus cells (lane 6 and 7) but not in oocytes (lane 2 and 3) or  $\beta$ -actin primer (lanes 4 and 8).

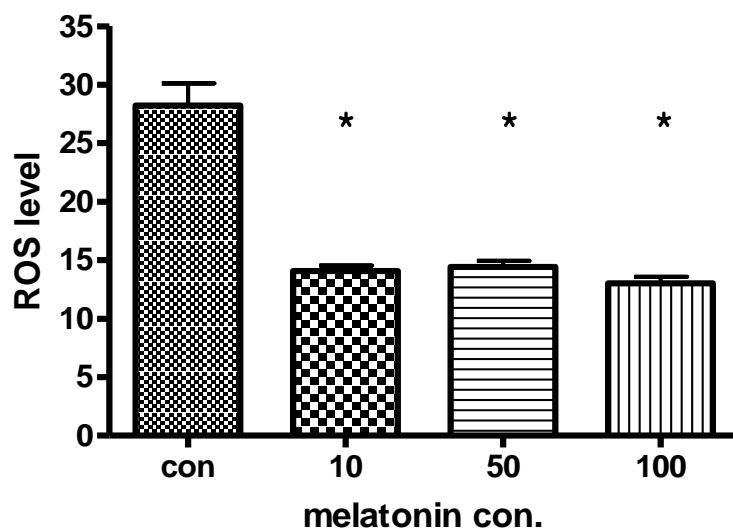


**Figure 4.** RT-PCR analysis of MT1 melatonin receptor mRNA expression in the porcine granulosa cells

DNA gel electrophoresis of first (lane 2) or second (lane 3) amplification products generated from porcine granulosa cells. Lane 1, DNA molecular weight marker (DNA-*Hae*IV). Using MT1 nested primers, a band of 461 bp corresponding to expected size for the amplified product of the MT1 melatonin receptor was obtained in granulosa cells (lane 2 and 3) or  $\beta$ -actin primer (lanes 4).

### 3.4 Measurement of ROS in porcine oocytes

In experiment 4, about 130 oocytes were used in four replicates to evaluate the effect of melatonin during IVM on the levels of ROS. ROS contents in oocytes matured in medium supplemented with melatonin, ranging from 13.0 to 14.4 pixels, was significantly lower in oocytes than those matured in control medium (28.2 pixels, Figure 5). Although the level of ROS was significantly reduced in melatonin-treated groups, there was no significant difference among any of the melatonin-treated groups.



**Figure 5.** Effects of four concentrations of melatonin on levels of ROS during IVM of porcine oocytes

The symbol (\*) indicates a significantly difference.

## 4. Discussion

The present study demonstrates that supplementing melatonin to IVM medium enhances oocyte maturation rate and embryo development. We found that melatonin improved the maturation rate of porcine oocytes during maturation in TCM-199. The rate of further development in blastocysts was also significantly higher when melatonin-treated oocytes were activated and further cultured in a PZM-3 medium. These findings demonstrate that melatonin supplementation of IVM medium improved both nuclear and cytoplasmic maturation.

My study on embryonic development was based on parthenogenesis rather than *in vitro* fertilization or somatic cell nuclear transfer to assess oocyte competence for embryonic development. The parthenogenetic activation of the oocytes can be used to evaluate the developmental competence of oocytes *in vitro* without confounding factors from the sperm [96] and a variable factors introduced during other procedures *in vitro*. Furthermore, parthenogenetic activation is relevant to cloning research, because artificial activation of an oocyte is an essential component of nuclear transfer protocols [156]. In mammals, parthenogenesis can yield viable offspring provided that parent-specific imprints regulating gene expression are overcome to permit the formation of a functional placenta [157]. Even without such manipulations, parthenogenesis still provides a valuable measure of oocyte competence to initiate the developmental program since development to the blastocyst stage is not affected by an epigenetic imprinting [158]. Accordingly, it is commonly used to assess oocyte competence to support

early development following somatic or pronuclear transfer [159, 160]. When compared to *in vitro* fertilization, the method of parthenogenetic activation used in my study has previously been shown to yield similar rates of development to the blastocyst stage [161], however, similar to my observation, these investigators recorded a significant reduction in cell numbers of the embryos resulting from parthenogenesis.

Recent studies have demonstrated that melatonin enhances *in vitro* embryo development in different species. According to Ishizuka et al.[148], melatonin at a  $10^{-6}$  M concentration supports fertilization and early embryo development after *in vitro* fertilization in mice. In cows, Papis et al. [162] reported that a relatively short period of *in vitro* culture with melatonin may have significant effects on the final development rate and, to some extent, on a quality (cell number) of bovine pre-implantation embryos fertilized *in vitro*. In addition, Rodriguez-Osorio et al. [163] reported that melatonin at a  $10^{-9}$  concentration has a positive effect on porcine embryo cleavage rates and blastocyst total cell numbers and it has a possible protective effect against heat stress.

In mammals, melatonin and its receptor (MT1 and MT2) mRNA are expressed in various tissues. Two mammalian melatonin receptor subtypes have been reported, MT1A and MT1B, both of which are G-protein coupled receptors [164]. The MT1A gene has been mapped in human, mice, sheep, pigs and cattle [155, 165]. The MT1B gene has been mapped to chromosome 9 in pigs [166]. However, the cellular expression and the role of melatonin systems have never been investigated

in porcine ovaries. Here, I report the presence of melatonin receptor (MT1) on cumulus cells and granulosa cells from porcine ovarian follicles.

Although melatonin is considered an exclusively neuronal hormone, its receptors have been found in tissues outside the nervous system. In particular, the binding of labeled melatonin and the presence of melatonin receptors has been documented in the human ovary [167]. Specific localization of MT1 melatonin receptors at the cellular level has only been demonstrated in granulosa cells [167]. The potential effect of melatonin on the differentiation of granulosa cells has not yet been established. The presence of high levels of melatonin in human preovulatory follicular fluid suggests that this hormone influences human ovarian and reproductive function [168, 169].

The present results show the presence of MT1 melatonin receptor transcripts in porcine cumulus cells and granulosa cells. The amount of transcripts seems to be lower compared with the amount of  $\beta$ -actin transcript. Presently, known mechanisms of melatonin action fall into three categories: receptor-mediated, protein-mediated and non-receptor-mediated effects [170]. Receptor-mediated melatonin events involve both membrane and nuclear binding site [171]. The effects of melatonin on reproductive function are thought to be mediated by G protein-coupled MT1 receptors in porcine. It is generally thought that melatonin influences reproductive functions at the level of the brain and pituitary. However, the presence of high levels of melatonin in follicular fluid [168] and its binding sites in the granulosa cells [172, 173] suggest that melatonin exerts its effects via a

receptor-mediated event at the level of the ovary. Some effects could involve modulation of steroidogenesis [174, 175] and luteolysis. The expression of receptor mRNA from cumulus cells and granulosa cells implies that melatonin may act directly on the oocyte for maturation.

As shown in Experiment 4, the present study also demonstrates that melatonin has antioxidative effects during *in vitro* maturation of oocytes. The beneficial effects of melatonin against oxidative stress and related damage in animals and humans, could improve mitochondrial function by counteracting mitochondrial oxidative stress [170].

The ROS may originate from embryo metabolism and/or embryo surroundings [130] and are detrimental to embryonic development [86, 176, 177]. The two-cell embryo block observed in mouse embryos was associated with a rise in ROS [86]. It has been suggested that the increase in ROS concentration during embryos cultured may lead to embryo apoptosis [161, 177]. Among ROS,  $H_2O_2$  plays a major role in apoptosis [178, 179].

Based upon results of the present study, I conclude that supplementation of IVM medium with melatonin could enhance porcine oocyte maturation and further embryonic development. Reduction of ROS could be an aspect of mechanism by which melatonin exerts its beneficial effects during oocyte maturation



## **Chapter 2. Quercetin improves *in vitro* development of porcine oocytes by decreasing reactive oxygen species levels**

### **1. Introduction**

Pigs have become useful animal models for organ xenotransplantation and as human disease models. Due to these important roles, *in vitro* culture of porcine oocytes is a very crucial process in the study of pre-implantation embryo development and in the production of transgenic animals. To increase the success rates of oocytes maturation and blastocyst formation *in vitro* and to improve cloning efficiency after embryo transfer, it is essential to produce high-quality matured oocytes. Many research studies have attempted to improve the production of high-quality oocytes by technical improvements across all stages of pre-implantation development. Among the most important factors influencing the developmental potential of embryos produced *in vitro* are the culture conditions including the external oxygen concentration that affect both oocyte maturation and embryo development [180].

ROS are endogenously produced by oocytes and embryos during *in vivo* development and IVC. Examples of ROS include oxygen ions and peroxides, particularly superoxide anions and hydroxyl radicals that are generated during the

process of oxygen reduction. Environmental stress can dramatically increase ROS levels. This may result in significant damage to cell membranes and plays a role in apoptosis. Additionally, the over-production of intracellular ROS in mammalian embryos during IVC is generally thought to be detrimental to embryo development [129]. Because pre-implantation embryos are particularly sensitive to ROS damage [130], the deleterious effect of ROS result in developmental inhibition [161]. In previous studies of pigs, IVF embryos with increased levels of ROS were found to have low developmental competence and increased DNA fragmentation [181]. Many reports have focused on overcoming these detrimental effects of ROS on embryo development. For example, reducing oxygen tension [116, 182] or treatment with antioxidants [183] during IVM or IVC improves embryo development. Therefore, it is important to protect oocytes against oxidative stress during IVM. One approach is to supplement the medium with antioxidant compounds during IVM.

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one) is a plant-derived flavonoid mainly found in fruits and vegetables. In mammals, flavonoids exert various biological and pharmacological effects [184]. Several studies have indicated that quercetin may have anti-inflammatory and antioxidant properties [185] due to its free radical scavenging and metal chelating properties [186]. In addition, this compound exerts a potent antioxidant effect. On the other hand, quercetin can also elicit pro-oxidant effects [187]. A relationship has been demonstrated between the free radical scavenging activity and anti-carcinogenic and anti-inflammatory properties of quercetin [188, 189].

Studies have been performed to evaluate the physiological functions and biological activities of quercetin in human and animal [190, 191]. However, there is limited information available on the effect of quercetin on oocyte maturation and embryonic development in pigs. Quercetin has been found to exhibit both estrogenic and anti-estrogenic effects in pigs *in vitro*, suggesting that this compound has different potential impacts on reproductive function [192].

Given this information, the objective of the present study was to examine the possible effect of quercetin supplementation during IVM and IVC on pig oocyte maturation and the developmental competence of parthenogenetically activated embryos. For this, I monitored the nuclear maturation and ROS levels of porcine oocytes, embryo cleavage and blastocyst formation of parthenogenetic embryos.

## 2. Materials and Methods

### 2.1 Experimental design

In order to identify the effective quercetin concentrations for improving oocyte maturation (Experiment 1), IVM medium was supplemented with four concentrations (0, 1, 10 or 50 µg/ml) of quercetin during the entire 44 hr maturation period. For experiment 2, I evaluated the effects of the same four concentrations of quercetin in IVM medium on the parthenogenetic development of embryos. In experiment 3, I assessed the effects these concentrations of quercetin in the IVM medium on the ROS levels in oocytes to assess the effect of antioxidant of quercetin.

### 2.2 Oocyte collection and *in vitro* maturation

Detailed protocols were described in general methodology (Part II). After culture of 44 hr, the meiotic maturation was determined by evaluating for the presence of the polar body as described in general methodology. After each maturation period, TALP medium was used as oocyte washing medium and denuding medium in this chapter.

### 2.3 Parthenogenetic activation of matured oocytes and *in vitro* culture

At 44 hr of IVM, metaphase II oocytes were parthenogenetically activated as described as general methodology (Part II). Following experimental design, A group of approximately 20 to 30 parthenogenetically activated oocytes were cultured in 500 µl PZM-5 for 7 days at 39 °C in a humidified atmosphere with 5%

CO<sub>2</sub> and 5% O<sub>2</sub>. The cleavage rate and blastocyst formation rate were checked at 48 and 168 hr of IVC, respectively. The quality of blastocysts was assessed by Hoechst staining as general methodology.

#### 2.4 Measurement of ROS level

Oocytes were sampled at 44 hr after IVM to determine intracellular ROS levels as previously described in general methodology (Part II).

#### 2.5 Statistical analysis

All statistical analyses were performed using Prism software (ver. 4.0; GraphPad, USA). A one-way ANOVA followed by a Tukey test was used to measure statistical differences among groups. *p*-values < 0.05 were considered to be statistically significant. Data are expressed as the means ± S.E.M..

### **3. Results**

#### **3.1 Effect of quercetin on porcine oocyte nuclear maturation**

About 782 oocytes were used for five replicate trials to evaluate the effects of quercetin on nuclear maturation during IVM. The polar body extrusion rate was not significantly different among the controls and groups treated with 1 or 10 ug/ml quercetin. In contrast, this rate was significantly lower ( $p < 0.05$ ) for the oocytes given 50 ug/ml quercetin ( $50.7\% \pm 1.9$ ) compared to the control ( $81.23\% \pm 1.0$ ) and other groups (Table 6).

**Table 6.** Effects of quercetin supplementation during IVM on the frequency of polar body extrusion

Concentration (ug/ml)	Oocytes, n	Oocytes with polar body extrusion, n	Polar body extrusion rate (% $\pm$ S.E.M.)
0 (Control)	192	156	81.23 $\pm$ 1.0 <sup>a</sup>
1	202	171	84.32 $\pm$ 2.4 <sup>a</sup>
10	197	160	80.97 $\pm$ 2.5 <sup>a</sup>
50	191	96	50.70 $\pm$ 1.9 <sup>b</sup>

Polar bodies were counted by Hoechst staining after 44 hr maturation *in vitro*.

<sup>a, b</sup> : Within the same column, values with different superscripts were significantly different (P<0.05).

### 3.2 Effects of quercetin on subsequent development of porcine oocytes *in vitro*

A total of 672 oocytes underwent IVM in media supplemented with four concentrations of quercetin. The oocytes were parthenogenetically activated and *in vitro* development was evaluated. Quercetin supplementation had no effect on the first cleavage frequency or cell number per blastocyst (Table 7). However, a significantly greater ( $p<0.05$ ) proportion of blastocysts developed into oocytes when the IVM medium was supplemented with 1 ug/ml quercetin. Addition of 1 ug/ml quercetin to the IVM medium improved the frequency of blastocyst development, but both the cleavage frequency and the rate of blastocyst formation of oocytes treated with the highest concentration of quercetin (50ug/ml) were significantly depressed compared to all other groups (Table 7).



**Table 7.** Effects of quercetin treatment of porcine oocytes on subsequent development *in vitro*

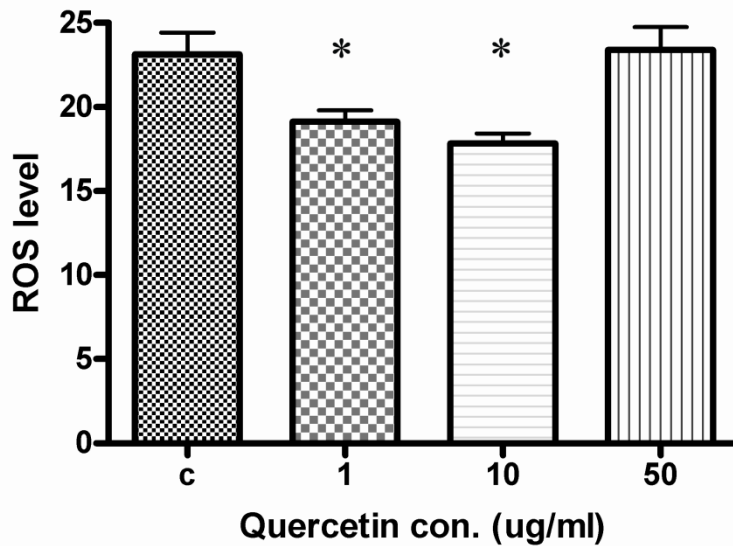
Concentration (ug/ml)	Oocytes examined, n	Cleavage, n (%) <sup>*</sup> ≥ 2 cell	Blastocysts, n (% ± S.E.M.)	No. of cells per blastocyst (means ± S.E.M.)
0 (Control)	191	123 (64.69)	18 (9.82±2.2) <sup>a</sup>	31.80 ± 2.6
1	171	135 (65.58)	32 (15.81±1.7) <sup>b</sup>	34.10 ± 2.0
10	160	105 (54.71)	17 (8.46±1.8) <sup>a</sup>	35.00 ± 3.0
50	96	48 (33.99) <sup>a</sup>	5 (3.46±1.6) <sup>c</sup>	31.40 ± 1.4

Percentages are based on the number of oocytes examined.

<sup>a, b, c</sup> : Within the same column, values with different superscripts were significantly different (P<0.05).

### 3.3 Measurement of ROS in porcine oocytes

For experiment 3, about 230 oocytes were used in four replicate trials to evaluate the effect of quercetin on the levels of ROS during IVM. The relative H<sub>2</sub>O<sub>2</sub> contents of oocytes matured in medium supplemented with 1 or 10 ug/ml quercetin ranged from 13.0 to 14.4 pixels, and were significantly lower than those in oocytes matured in control medium (Fig.6; 28.2 pixels,  $p<0.05$ ). However, the relative H<sub>2</sub>O<sub>2</sub> contents were not significantly different between the control and oocytes treated with 50 ug/ml quercetin.



**Figure 6.** Effects of four concentrations of quercetin on levels of ROS during IVM of porcine oocytes

The oocytes were stained with DCHFDA following maturation. Fluorescence intensity representing the concentrations of ROS generated in the mature oocytes was recorded using a cooled CCD camera attached to a fluorescence microscope. DCHFDA staining intensity was normalized to each imaged oocytes area (pixels).

\* indicates a significantly difference ( $p < 0.05$ )

## 4. Discussion

In the present study, the effects of quercetin as an antioxidant during IVM and IVC on oocyte maturation and embryonic development after parthenogenetic activation were examined, and the intracellular levels of ROS were measured. My results demonstrate that quercetin enhances the *in vitro* development of porcine oocytes. In quercetin-treated oocytes, ROS concentrations were significantly lower after maturation. Additionally, the rate of blastocyst formation was significantly higher in quercetin-treated oocytes that were activated and further cultured compared to the control group. On the other hand, excessive doses of quercetin were detrimental to oocytes and embryos without reducing the levels of ROS.

In recent years, interest in quercetin has gradually increased along with other flavonoids due to its health promoting activities that likely result from antioxidant effects in humans and animals. Nevertheless its overall biological impact of quercetin remains controversial, mostly due to the limited information about its bioavailability, endogenous dynamics and relative contribution of different types of conjugates. Some studies have showed that quercetin can mediate cancer cell apoptosis [193, 194]. Furthermore, these investigations have indicated that quercetin can selectively induce apoptosis of cancer cells and not normal cells. Other research has shown that quercetin can protect against oxidative stress by decreasing ROS generation through its antioxidant activity in normal human cells [195, 196]. My study demonstrates that quercetin at the optimal concentration acts

as an antioxidant and positively affects the maturation of porcine oocytes and *in vitro* embryonic development.

ROS are generated by embryonic metabolism or in the surrounding environment during IVC [197], and are detrimental to embryonic development [177]. Increased ROS levels are associated with the two-cell embryo block in mice [86]. It has been suggested that the increased ROS concentrations may lead to apoptosis during embryo culture [161]. Among different ROS, H<sub>2</sub>O<sub>2</sub> at high concentrations induces apoptosis [178]. Therefore, this study was conducted to monitor the level of H<sub>2</sub>O<sub>2</sub> within oocytes to indirectly assess ROS toxicity. I also examined parthenogenetic embryonic development to evaluate oocyte competence rather than *in vitro* fertilization or somatic cell nuclear transfer. The reason for this was because parthenogenetic activation can be used to evaluate oocyte developmental competence *in vitro* without confounding factors from sperm and a variety of other reagents introduced during *in vitro* procedure. Furthermore, parthenogenetic activation is relevant to cloning research because artificial oocyte activation is an essential component of nuclear transfer protocols [197].

The concentrations of quercetin used in this study (1, 10 and 50ug/ml) were selected based on previous results from a study of porcine granulosa cells [192]. In this study, 50ug/ml quercetin was found to inhibit progesterone production, modifies estradiol-17 $\beta$  production, and interferes with angiogenesis in granulosa cells by inhibiting vascular endothelial growth factor production, implying that quercetin may have a negative influence on ovarian physiology. Despite the

detrimental effect of quercetin on embryos, my study showed that treatment with adequate concentrations of quercetin (1 and 10 ug/ml) improved embryonic development, but it was not clear whether quercetin directly affects embryo development by decreasing ROS toxicity. I also found that rates of oocyte maturation and blastocyst formation were substantially reduced with a high concentration of quercetin (50ug/ml), although the expansion and total cell number of blastocysts were not adversely affected, demonstrating that quercetin has dose-specific effects on oocytes.

I suggest that the reduction of oocyte maturation and blastocyst formation rates with this concentration of quercetin may result from unresponsive signaling to oocytes and embryos or direct embryo toxicity owing to excessive levels of flavonoids. Some groups have reported toxic effects of other flavonoids on embryos from different species such as genistein [198], puerarin [199], ginkgolides [200] and purple sweet potato anthocyanins [201]. In contrast, several investigations have shown that supplementation of porcine IVM medium with antioxidants such as selenium, vitamin E and ascorbic acid decreases ROS levels while enhancing the developmental competence of IVF embryos and parthenotes [89, 181, 202]. Another study recently demonstrated that treatment with anthocyanin, a type of flavonoid, in IVM media improves the developmental competence of cloned pig embryos, most likely by increasing glutathione synthesis and reducing ROS levels [95]. Compared to a previous study conducted with other antioxidants, the effects of quercetin on the maturation rate, blastocyst formation rate and ROS generation rate in oocytes I observed were less potent.

In my investigation, no beneficial effect of quercetin treatment on the first polar body extrusion rate during IVM was found even though quercetin effectively reduced ROS levels. However, a beneficial effect of quercetin treatment at less than 10ug/ml was observed during subsequent culturing to the blastocyst stage. Therefore, it may be that the antioxidant effect of quercetin on oocytes was maximized during subsequent IVC, and thus, as suggested by previous studies and the present investigation, ROS may play a pivotal role in regulating oocyte maturation and embryonic development.

In conclusion, treatment of porcine oocytes with quercetin had a significant effect on embryonic development. At low levels, quercetin reduced intracellular ROS levels but was detrimental at high concentration. It is not clear whether this low level of quercetin is optimal in pigs. In addition, the rate of blastocyst formation was significantly increased by quercetin treatment whereas there was no increase in the number of blastocyst cells. Further studies are needed to determine the optimal concentration of quercetin and to ascertain the beneficial effects of this compound on pig embryo development.

# **Chapter 3. Effect of antioxidant flavonoids (Quercetin and Taxifolin) on *in vitro* maturation of porcine oocytes**

## **1. Introduction**

Increasing the efficiency of systems for *in vitro* production of porcine embryos is very important because pigs have high biomedical value for areas such as xenotransplantation and as models for stem cell research [203, 204]. However, despite intensive efforts, the yield and quality of IVM oocytes and embryos derived from them are still low compared with *in vivo* produced embryos. Improvements can be made by altering the culture conditions for oocyte maturation and embryo development, including the external oxygen concentration [180].

Oxidative stress originating from high external oxygen concentration can produce ROS [177], which may be responsible for damaging embryos and inducing early embryonic developmental blocks [130]. Therefore, supplementing maturation and culture media with antioxidants such as  $\beta$ -mercaptoethanol, cysteine and cysteamine can help to protect against defective embryo development [205, 206] . The glutathione (GSH) is intracellular free thiol compound, involved in protecting cell from ROS toxicity and regulates the intracellular redox balance. So, intracellular increase of GSH during *in vitro* process has beneficial effects on



porcine embryo development [36]. Also, supplementation of some antioxidants into IVM media could stimulate the synthesis of intracellular GSH, then improve embryo development in pig [205].

Flavonoids are a class of plant secondary metabolites and are most commonly known for their antioxidant activity *in vitro* [207]. Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one) and Taxifolin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one) are plant-derived flavonoids mainly found in fruits and vegetables. It has been reported that quercetin and taxifolin have anti-oxidative, anti-mutagenic and anti-inflammatory activities [186, 189, 208, 209] due to free radical scavenging. Also, Taxifolin is not mutagenic and has low toxicity compared to the related compound quercetin [210]. In previous chapter, I demonstrated that exogenous quercetin is beneficial for nuclear maturation during IVM and subsequent embryo development by reducing ROS levels [211].

However, little information is available on the effect of flavonoids on oocyte maturation and embryonic development in pigs. The objective of this chapter was to examine the effect of quercetin and taxifolin treatment during IVM and IVC on oocyte maturation and the development of parthenogenetically activated (PA) embryos. To this end, I observed nuclear maturation of oocytes, embryo cleavage and blastocyst formation of PA embryos, as well as intracellular levels of GSH and ROS in pig oocytes and embryos.

## 2. Materials and Methods

### 2.1 Experimental design

In order to determine effective concentrations of taxifolin for improving oocyte maturation (Experiment 1), it was included in the IVM medium (TCM-199) at four concentrations (0, 1, 10 or 50 µg/ml) during the entire maturation culture period of 44 hr. In experiment 2, I evaluated the effects of including quercetin or taxifolin in the IVM medium compared with a non-treated control group on the parthenogenetic development of embryos. In experiment 3, I assessed the effects of these quercetin and taxifolin concentrations in the IVM medium on the ROS and GSH levels in oocytes and embryos. Also, the effects of quercetin and taxifolin on maturation as phytoestrogen were assessed thorough hormone (P4 and E2) radioimmunoassay (experiment 4).

### 2.2 Oocyte collection and *in vitro* maturation

Detailed protocols were described in general methodology (Part II). After culture of 44hr, the meiotic maturation was determined by evaluating for the presence of the polar body as described in general methodology. After each maturation period, TALP medium was used as oocyte washing medium and denuding medium in this chapter.

### 2.3 Parthenogenetic activation of matured oocytes and *in vitro* culture

At 44 h of IVM, metaphase II oocytes were parthenogenetically activated as described as general methodology (Part II). Following experimental design, a

group of approximately 20 to 30 parthenogenetically activated oocytes were cultured in 500  $\mu$ l PZM-5 for 7 days. The cleavage rate, blastocyst formation rate and the quality of blastocysts was checked as described in general methodology.

#### 2.4 Measurement of intracellular GSH and ROS levels

Oocytes were sampled after 44 hr of IVM and 2 d of IVC to determine intracellular GSH and ROS levels using the DCHFDA and CellTracker Blue CMF2HC methods previously described as general methodology.

#### 2.5 Effect of flavonoid on cumulus cell steroidogenesis

IVM media of both the first and second day of culture were collected, centrifuged at 1500 rpm for 3 min and the supernatants were stored at -20 °C until assayed for progesterone (P4) and estradiol-17 $\beta$  (E2) by validated radioimmunoassay. All samples were analyzed with assistance of the Neodin Veterinary Laboratory (Seoul, Republic of Korea; <http://www.vetlab.co.kr>).

#### 2.6 Statistical analysis

All statistical analyses were performed using Prism4 software (GraphPad, USA). One-way ANOVA was used to determine significant differences in the data followed by a Tukey test to determine statistical differences among groups. Significant differences among the treatments were determined when  $P < 0.05$ . Data are expressed as means  $\pm$  S.E.M..

### **3. Results**

#### **3.1 Effect of taxifolin on porcine oocyte nuclear maturation**

A total of 746 oocytes were used in five replicates to evaluate the effects of taxifolin on nuclear maturation during IVM. The polar body extrusion rate was not significantly different among the control and the treatment groups at 1 or 10 ug/ml, but it was significantly lower ( $P < 0.05$ ) in the group containing 50ug/ml taxifolin ( $59.2\% \pm 7.9$ ) compared with the control ( $80.5\% \pm 3.5$ ) and the other treatment groups (Table 8).

**Table 8.** Effects of taxifolin during oocyte IVM on the frequency of first polar body extrusion (nuclear maturation)

Concentration (ug/ml)	Oocytes, n	Oocytes with polar body extrusion, n	Polar body extrusion rate (% $\pm$ S.E.M.)
0 (Control)	181	146	80.54 $\pm$ 3.5 <sup>a</sup>
1	192	162	84.43 $\pm$ 2.6 <sup>a</sup>
10	194	152	78.50 $\pm$ 3.2 <sup>a</sup>
50	179	107	59.21 $\pm$ 7.9 <sup>b</sup>

Polar bodies were counted by Hoechst staining after 44 hr maturation *in vitro*.

<sup>a, b</sup> Within a column, values with different superscripts are significantly different (P<0.05).

### 3.2 Effects of quercetin and taxifolin on PA embryo development

Based on the results from Experiment 1 and my previous study [211], 1 ug/ml of quercetin or taxifolin were used in Experiment 2. A total of 800 oocytes that underwent IVM in media supplemented with quercetin or taxifolin were parthenogenetically activated in nine replicates. Quercetin or taxifolin had no effect on the first cleavage frequency or the cell number per blastocyst (Table 9). However, a significantly greater ( $P<0.05$ ) proportion of blastocysts developed from oocytes when the IVM medium was supplemented with 1 ug/ml quercetin (Table 9). Further treatment with quercetin or taxifolin during IVC did not have any stimulatory effect on embryonic development (data not shown).

**Table 9.** Effects of quercetin treatment of porcine oocytes on subsequent development *in vitro*

Treatment (1 ug/ml)	Oocytes examined, n	Cleavage, n (%) ≥2 cell	Blastocysts, n (% ± S.E.M.)	No. of cells per blastocyst (means ± S.E.M.)
Control	265	182 (68.80)	44 (16.84±2.0) <sup>a</sup>	49.75 ± 3.7
Quercetin	266	188 (70.87)	64 (24.30±2.3) <sup>b</sup>	48.00 ± 2.6
Taxifolin	269	198 (73.58)	47 (17.74±1.9) <sup>a</sup>	51.86 ± 4.5

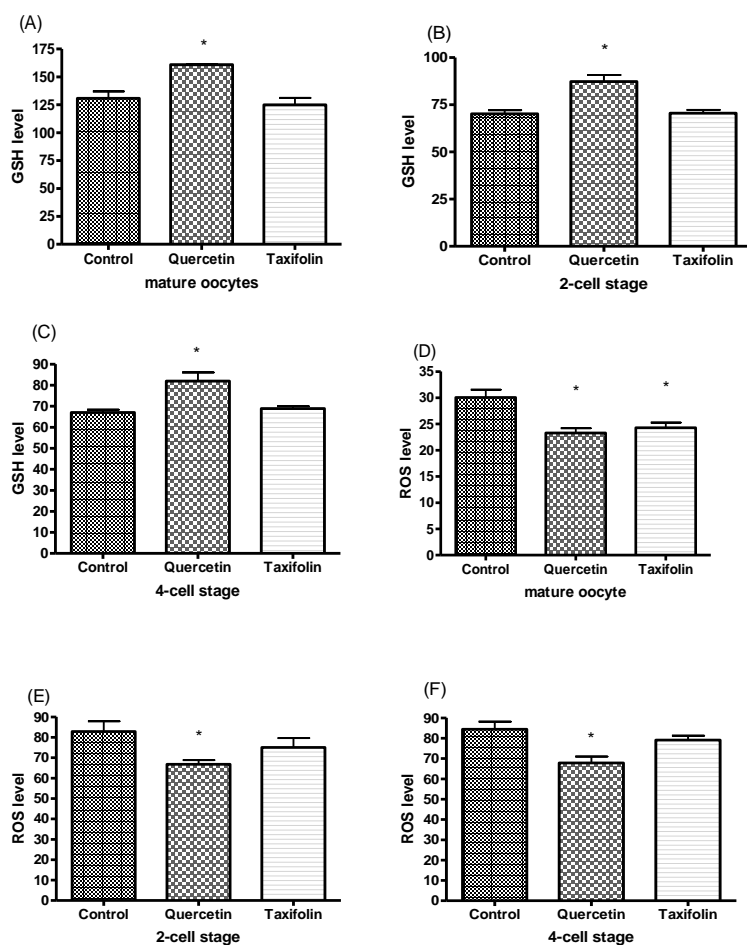
Percentages are based on the number of oocytes examined.

<sup>a, b</sup>Within a column, values with different superscripts are significantly different (P<0.05).

### 3.3 Intracellular levels of ROS and GSH in porcine oocytes and embryos

In Experiment 3, 430 oocytes were used in four replicates to evaluate the effect of quercetin and taxifolin during IVM and IVC on the levels of ROS and GSH. Quercetin increased intracellular GSH levels and decreased ROS generation in matured oocytes and in cultured embryos ( $p < 0.05$ ). Taxifolin also reduced the ROS levels in matured oocytes ( $p < 0.05$ ) but not in cleavage stage embryos, while GSH levels were not significantly different in oocytes or embryos compared with the control group (Figures 7).





**Figure 7.** Measurement of intracellular GSH and ROS levels in IVM oocytes and parthenogenetically activated embryos. The symbol (\*) indicates a significant difference ( $P < 0.05$ ). Values shown on the Y-axis are number of pixels.

### **3.4 Effect of flavonoid on cumulus cell steroidogenesis**

Basal steroid production by cumulus cells after 22 and 44 hr of culture is shown in Table 10. Progesterone and estradiol levels in culture media of oocytes incubated with and without flavonoid (QT or TF) were investigated. Both concentration of flavonoid (QT and TF) didn't significantly have differences.

**Table 10.** Effects of Flavonoid (QT or TF) on cumulus cell steroidogenesis

treatment	Estradiol (pg/mL)		Progesterone (ng/ml)	
	22hr	44hr	22hr	44hr
Control	1326.5	1161.7	85.0	323.7
Quercetin	1206.3	1144.3	80.7	295.3
Taxifolin	1223.8	1168.0	75.8	395.2

E2 and P4 production by cumulus cells after 22 and 44hr of *in vitro* maturation of oocytes in absence (control) or in presence of 1 ug/ml quercetin or 1 ug/ml taxifolin. Data represent mean of at least four replicates.

## 4. Discussion

In this study, effects of the antioxidants (quercetin and taxifolin) were examined during oocyte maturation and embryonic development following parthenogenetic activation, and on intracellular levels of ROS and GSH. Although porcine PA embryos cannot develop beyond 29 days of gestation *in vivo* [212], they may be good models to evaluate effects of exogenous factors during *in vitro* embryonic development. My results showed that the rate of PA blastocyst formation from quercetin-treated oocytes was significantly higher than in the control group and in the taxifolin-treated group. However, quercetin did improve the cleavage rate or total cell number of blastocysts compared with the other groups. Supplementing the IVM medium with taxifolin did not improve nuclear maturation, but was effective in reducing ROS levels in matured oocytes. The inclusion of quercetin but not taxifolin in the IVM medium increased PA blastocyst formation, presumably because quercetin reduced ROS and increased intracellular GSH more effectively than the taxifolin treatment. However, I have found no beneficial effect of quercetin or taxifolin treatment on blastocyst formation when applied only during IVC (data not shown). It is not clear whether improved embryonic development observed after treatment of oocytes with quercetin or taxifolin was due to a direct action of quercetin or taxifolin on embryos or to the reduction of ROS toxicity by increasing GSH.

ROS may originate from embryo metabolism and/or the embryo environment, and are detrimental to embryonic development [176, 177]. Many antioxidants can

alleviate oxidative stress during reproductive processes, and can enhance embryonic development *in vitro*. In pigs, as in other mammals, several antioxidants have been used as supplements in culture media to enhance embryonic development [197, 213], but little research has been done with flavonoids, which are well-known and powerful natural antioxidants. To the best of our knowledge, this is the first study investigating quercetin or taxifolin effects on early embryonic development *in vitro* in pigs. My data indicate that the optimal amount of flavonoid is concentration-specific, and while lower concentrations elicit no observable responses, excessive levels could be toxic to the oocyte.

Due to their structural similarity with estrogen, several flavonoids, including genistein and daidzein, also interact with the estrogen receptor to mediate their activity and thereby act as weak or moderate phytoestrogens [214, 215]. Female mice treated neonatally with genistein showed multi-oocyte follicles, lacked regular estrous cyclicity and showed implantation failure, although ovulated oocytes were developmentally competent [216]. Therefore, to confirm treatment effects of flavonoids as phytoestrogens, I assayed for P4 and E2 by radioimmunoassay during the oocyte maturation period (44hr). For P4 and E2, steroid concentrations are expressed steadily without differences among groups (control, 1 $\mu$ g/ml quercetin- and taxifolin- treated group) (Table 3). Maybe it thought that quercetin and taxifolin in this concentration is not effective in changing basal estradiol-17 $\beta$  secretion as phytoestrogens, and this finding is in agreement with the results of other flavonoids obtained by Galeati et al [214] in

porcine granulosa cells, showing that these flavonoids in this concentration may affect very weakly on oocytes or granulosa cells as phytoestrogens.

Previous studies reported that addition of high concentrations of antioxidants to the IVM medium decreased the rate of blastocyst formation compared to treatment with low concentrations [36, 217], suggesting that the proper concentration of an antioxidant can contribute to the generation of high quality embryos. In my experiments, treatment with 50ug/ml taxifolin was detrimental to oocyte maturation, which is consistent with the result using quercetin in my previous study [211]. One study reported that quercetin at 50ug/ml inhibited progesterone production by granulosa cells, altered estradiol-17 $\beta$  production, and interfered with angiogenesis by inhibiting VEGF production, so quercetin may have a negative influence on ovarian physiology [192]. Both quercetin and taxifolin in high concentration (at least 50ug/ml) may have detrimental effects to oocytes by influencing culture environments.

I compared effects of quercetin and its analogue taxifolin, both at 1 ug/ml, on embryo development after parthenogenetic activation (Experiment 2). Previous research has shown that using antioxidants during oocyte maturation increases cytoplasmic maturation and leads to higher rates of IVF and embryo development [205]. In my study, the antioxidant quercetin applied during IVM increased intracellular GSH levels and improved blastocyst development, which implies enhancement of cytoplasmic maturation. However, no beneficial effect of taxifolin treatment during IVM was found on first polar body extrusion rate, showing that it

could not affect nuclear maturation even though it effectively reduced ROS levels. Also, my observations that antioxidants, specifically quercetin, applied during oocyte maturation increases blastocyst formation, are in agreement with previous findings [95, 218-220].

In conclusion, treatment of porcine oocytes with the quercetin, a type of flavonoid, had a significant positive effect on embryonic development and reduced ROS generation by increasing intracellular GSH levels at low concentrations, but it was detrimental at high concentrations. It is not clear whether this concentration of quercetin is optimal in pigs. Therefore, further studies are needed to determine the optimal concentration of quercetin and to ascertain its beneficial effects on further development of pig embryos.

**PART V**

**GENERAL CONCLUSION**



In this study, for improving the maturation and embryo developmental efficiency of porcine oocytes, the effect of different oxygen tension as culture condition on IVM and IVC of porcine oocytes was investigated and the treatment effects of antioxidants such as melatonin, quercetin and taxifolin, on oocyte maturation and comparative effects of different oxygen concentration on oocytes culture condition were estimated.

On culture of the different oxygen concentration (5 and 20%), because blastocyst formation rate was estimated higher on culture condition of low oxygen concentration, low oxygen concentration indicated beneficial effect from oxygen toxicity on *in vitro* culture. According to analysis of differences in gene expression between cumulus-oocytes complexes cultured under 5 or 20% oxygen, each oxygen condition altered the expression of genes in different patterns. Specially, it showed that gene concerned antioxidant response was upregulated in the high oxygen condition. It can be concluded the low oxygen concentration may alter the expression of multiple genes related to oocyte competence and improves embryo development.

Supplements of maturation media with 10ng/ml melatonin improved the maturation rate and developmental competence. Oocyte cultured with melatonin generated lower ROS levels compared to others. Also we detected a local expression of melatonin receptor I on surface of the cumulus and granulosa cells through the presence of MT1 melatonin receptor transcript. These results showed

the exogenous melatonin works effectively on porcine oocytes maturation by working as a direct antioxidants or indirectly binding to receptors.

As plant-derived flavonoids, quercetin and taxifolin have an antioxidant property, I investigated the treatment effect of these flavonoids on embryo development. The result of quercetin and taxifolin treatment on maturation media showed that quercetin at the 1 $\mu$ g/ml has beneficial effect on developmental competence, but there is not significant effect on taxifolin treatment. Also, oocytes treated with quercetin had significantly lower levels of ROS and higher level of GSH. In this concentration, quercetin has not worked as phytoestrogens. Based on these findings, I concluded that exogenous quercetin reduces ROS levels during culture process and is beneficial for subsequent embryo development.

In conclusion, all data showed that because oxygen toxicity generated from culture process can cause detrimental effect on oocyte development, low oxygen tension during embryo culture have beneficial effect on embryo development and supplementation of antioxidant into the culture media may improve developmental competence of porcine oocyte *in vitro* by reducing ROS levels.

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## 요약 (국문초록)

바이옰�학 연구에 있어 돼지의 유용한 특성으로 인해 돼지 배아의 체외생산은 매우 중요한 단계로 여겨진다. 그러나 아직까지 돼지의 체외배아 생산율은 체내에 비해 낮은 편이다. 체외 배양시 발생하는 ROS 와 같은 산소독성은 낮은 생산율을 야기하는 다양한 원인중의 하나이다. 본 연구의 목적은 난자의 체외배양간 산소농도를 달리하여 산소독성이 야기될 수 있는 배양조건을 개선하고 더불어 배양배지에 항산화제를 처리함으로써 돼지 배아의 체외 생산율을 높이기 위함이다. 이러한 목적을 위해 본 연구는 체외 성숙 및 배양에 다른 산소농도 (5% 와 20%) 상황하에서 난자의 발달 효과 및 유전자 발현 현상을 검증하고, 돼지 난자에 멜라토닌과 몇몇 플라보노이드 (퀘세틴, 탁시폴린)을 각각 처리하여 체외발달에 대한 효과를 검증하였다.

우선, 다른 산소농도 (5% 와 20%)하에서 돼지난자를 성숙시키고 배양시켰을 때, 난자의 핵 성숙률에는 별다른 차이를 보이지 않았으나 20% 산소농도에서 성숙시킨 그룹을 5% 산소농도에서 체외 배양시 20% 산소농도에서 배양한 그룹보다 배반포 발달률이 유의적으로 향상되었다. 더불어 각 배양조건하에서 다양한 유전자의 mRNA 발현량 변화를 관찰하였을 때 낮은 산소하에서는 높은 글루코스 흡수작용과 혐기성 해당작용에 관련된 유전자가 높게 발현되었고 높은 산소하에서는 유사분열촉진 작용과 항산화작용에 관련된 유전자의 발현이 높았다.

따라서 난자를 20% 산소하에서 성숙시키고 낮은 산소하에서 배양하면 난자의 발달에 관련된 유전자 발현을 높여 난자의 발달을 유의적으로 증진시킬 수 있을 것이다.

배양환경에서 발생하는 ROS 독성을 방지하기 위해 난자의 체외성숙 배지에 몇몇 항산화제를 첨가하여 난자의 체외성숙 및 발달률을 검증하였다. 우선, 항산화제의 일종인 멜라토닌 호르몬을 성숙 배지에 첨가하였을 때, 10 ng/ml 의 처리 농도의 멜라토닌에서 난자의 성숙 및 배아 발달에 좋은 효과를 보였으며 ROS 발생 레벨도 낮추었다. 또한 멜라토닌의 발현 상태를 검증해본 결과 난자 주변의 난구 세포 및 과립막 세포에서 멜라토닌 수용체-1 유전자의 발현이 검증되었다. 결과적으로 외인성 멜라토닌은 돼지 난자의 체외성숙에 유용한 효과를 보였으며 이는 난구세포 표면 수용체와의 결합에 의한 간접적인 효과이거나 강력한 항산화제로서의 역할로 인한 직접적인 효과일 것으로 보인다.

또한 항산화제로서 플라보노이드의 일종인 퀘세틴을 성숙배지에 첨가하였을 경우 난자의 성숙에는 별다른 효과를 보이지 않았으나 지속 배양시 1 ug/ml 농도의 처리군에서 배반포 발달률이 유의적으로 향상되었다. 더불어 이 농도에서 난자는 낮은 ROS 발생레벨을 나타내었다. 그러나 고농도의 퀘세틴 처리는 오히려 난자 발달에 해를 끼치는 것으로 나타났다. 타키폴린 또한 플라보노이드로서 항산화 효과를 가지며 퀘세틴보다 독성이 낮은 것으로 알려져, 이후 두 인자를

성숙배지에 처리하여 그 효과를 비교하였다. 처리결과 50ug/ml 의 농도의 탁시폴린 처리군에서 난자에 유해한 효과를 보였다. 두 인자의 처리군을 비교한 결과 1 ug/ml 의 퀘세틴은 배반포 발달률 향상 효과를 보인 반면 1 ug/ml 의 탁시폴린은 유의적인 차이를 나타내지 않았다. 난자와 배아의 ROS 및 GSH 발생 레벨을 측정한 결과, 퀘세틴과 탁시폴린 처리그룹 모두 ROS 레벨을 낮추었으며 퀘세틴 처리그룹에서만 GSH 발생 레벨의 유의적인 차이를 나타내었다. 이 농도에서 퀘세틴과 탁시폴린 모두 식물성 에스트로젠으로서의 작용은 없었다. 결과적으로 퀘세틴과 같은 플라보노이드는 난자내의 ROS 발생레벨을 낮추어 체외발달에 효과적으로 작용한다.

이상의 결과를 종합하면, 체외배양간 산소농도의 감소는 돼지 난자의 체외배양 효율을 증대시킬 수 있었으며, 멜라토닌이나 퀘세틴과 같은 외인성 항산화제를 돼지 난자의 체외 배양 배지에 처리함으로써 배양기간 중에 발생하는 ROS 레벨을 낮추어 난자의 성숙 및 발달을 증진시킬 수 있었다.

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주요어 : 돼지 난자, 체외 성숙, 체외 배양, 항산화제, ROS

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